EFFECTS OF ALUMINIUM ON ARBUSCULAR MYCORRHIZAL SYMBIOSIS IN COWPEA PLANT GROWTH

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi could be of importance for plants growing in acid soils, but it much depends on their responses to soil acidity factors, such as excessive H^+ ion concentration and Aluminium (Al) toxicity. This study assessed separately the effects of Al and of low pH on the growth of *Gigaspora margarita* and its symbiotic function in the growth of cowpea (*Vigna unguiculata* L. Walp,).

Results of experiments using conventional pots containing growth media differing in pH (4.6 – 5.2) with sub-toxic levels of about 1 ppm soluble Al, or differing in soluble Al concentrations (1.1 – 11.9 ppm) at pH 4.7, showed that *Gi. margarita* was not affected by low soil pH tested, and so effectively increased growth of cowpea plants. However its function decreased with increasing Al in the soil.

Results of experiments using pots with 2 and 3 compartments, separated by 30 μm mesh that retains plant roots but lets fungal hyphae pass it to grow separately from the roots, showed that decreased fungal effectiveness was not related to percentage root colonization but to inhibited development of the external hyphae in the soil.

Excess soluble Al can inhibit directly different stages in the fungal life, including spore germination, germ tube growth and external hyphae growing from colonized plant roots. Al can also affect indirectly growth of the hyphae via effects on the host plant. Depression in growth of host plants induced by Al reduced the hyphal growth. However, increased growth of the host plants due to sufficient P nutrition increased the ability of the hyphae to deal with Al toxicity.

Exposure to high Al did not reduce the viability of the hyphae to initiate new colonization and to improve host plant growth as long as the plants were not exposed to high Al.

DECLARATION

I declare that this work contains no material, which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University Library, being made available for loan or photocopying.

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Agus Rohyadi

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To my late father,

who firstly taught me the importance of knowledge

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

The low productivity of acid soils in much of the world is well documented. Crop plants grown on these soils often suffer from adverse soil conditions resulting from the effects of heavy metal toxicity, nutrient deficiency, especially phosphorus (P), calcium (Ca) and magnesium (Mg), low organic matter and lack of water holding capacity (von Uexküll and Mutert, 1995). Aluminium (Al) is considered the major stress factor because of its solubilization under acidic conditions. Many studies have shown that excessive concentrations of Al ions in soil solution greatly reduce plant growth and yield (e.g. Baligar *et al.*, 1995). The phytotoxicity of the element is mainly expressed in two ways. Firstly, excess Al inhibits lateral root and root hair formation, creating a poor root system. Secondly, Al may limit the solubility and, therefore, the availability of some essential mineral nutrients, principally P, by fixing them in soils (Taylor, 1988). As a result, plants have difficulty in taking up nutrients and water in sufficient quantity, and consequently they are stunted.

From the agronomic point of view, Al toxicity remains a crucial problem, especially in many acid tropical soils, as there is no easy way to solve it. Cultivation of plant species or genotypes tolerant to Al has been

widely recommended (e.g Sanchez and Salinas, 1981; Myers and de Pauw, 1995). This is inexpensive and easy to practise, but has a constraint relating to the local availability of plant seed, particularly for farmers in many developing countries, whilst to breed and establish an Al tolerant genotype requires time, skills and resources (Myers and de Pauw, 1995). The alternative solution is the modification of soil conditions to make them more conducive to the growth of Al-sensitive genotypes in particular. A variety of methods can be applied such as liming, fertilizing or organic manuring (Barcélo *et al.*, 1996). Soil liming increases soil pH, thereby reduces soluble Al, and so improves plant performance. Nonetheless, it is rarely practised particularly in developing countries because of cost. As a result, there is a need to develop other appropriate solutions by considering soil characteristics. In this respect, many workers suggest manipulation of the potency of soil microorganisms, particularly glomalean fungi that form arbuscular mycorrhizal symbioses with plant roots.

Arbuscular mycorrhizas (AM) are very common in native and man-made ecosystems, and recognized as mutualistic symbioses. There is a wealth of information in the literature showing that AM fungi increase the growth of their host plant by supplying mineral nutrients, primarily immobile elements such as P, Zn and Cu and by inducing plant resistance to adverse soil conditions (Smith and Read, 1997). In these respects, the greatest effects of this symbiosis have been observed with plants that have coarse roots with a lack of root hairs or else a small root system, and/or are grown on soils with particularly low available P (Mosse, 1981; Manjunath and Habte,

1991). Recent work has demonstrated that the performances of several crop plants grown in acidic soil conditions were greatly improved by mycorrhizas; these include sorghum (Raju *et al.*, 1988), soybean, maize (Nurlaeny *et al.*, 1996), barley (Borie and Rubio, 1999), onion, cassava, cowpea (Yost and Fox, 1979) and banana (Rufyikiri *et al.*, 2000). This indicates that mycorrhizas can also be of great importance in acid soils, where P deficiency and poorly developed plant root systems, induced by excessive Al, are growth constraints.

Decreases in growth and function of AM fungi themselves at low pH have also been documented (Clark, 1997), but no conclusions have been made about the main cause, i.e. whether it was a high concentration of H^+ ions (low pH) *per se* or acidity-related factors. Some workers found that excessive Al reduced AM functioning in acid soils (Raju *et al.*, 1988; Borie and Rubio, 1999), but again the mechanisms are unknown. However, it appears that Al may impair the fungus directly, particularly during pre-symbiotic growth and indirectly by inhibiting host plant growth. Nevertheless, few good data support any of these explanations. Overall, many eco-physiological aspects of AM fungi in acid soils remain far from fully understood, so more investigations are required.

The study presented in this thesis was concerned with the functioning of AM symbiosis on plant growth in acid soil conditions, with particular interest in the interaction between AM fungi and Al toxicity.

The following section presents a brief review of literature, which covers the Al phytotoxicity and AM symbiosis and their effects on plant

growth, particularly in acidic soil conditions and in relation to Al toxicity. The review is based on results of studies published up to 2000 when the present study began. Papers published since then are incorporated in the discussion sections of relevant chapters and in the general discussion.

1.2 Review of literature

1.2.1 Aluminium in soil

Aluminium is the most abundant metal in the earth's crust and is found as a major element in almost all mineral soils, comprising up to 7.1% by weight of the soil (Wolt, 1994). It exists both in solid and soluble phases. Its solid phase is predominantly found as a component of various minerals such as gibbsite (hydrous oxides), feldspars, kaolinite and other clay minerals (alumino-silicates), jurbate (sulphates), and variscite (phosphates) (Ritchie, 1995). In soil solution, Al can be extensively hydrolyzed and polymerized, generating soluble forms that have complex speciation chemistry, both as monomeric and polymeric species (McBride, 1994). The concentrations and distributions of Al species in soil solution vary markedly with soil pH. Fig. 1.1 shows the relationship between solution pH and relative distribution and average charge of Al species. At very low pH, Al exists primarily as the hexa-aquo aluminium [$Al(H_2O)_6^{3+}$] species usually designated Al^{3+}, followed by other monomeric hydroxy-Al species such as $Al(OH)^{2+}$ and $Al(OH)_2^{+}$. At neutral or higher pH, Al species exist predominantly as $Al(OH)_3$ (an insoluble

form) and $Al(OH)_4^-$ (Robert, 1995). Polymeric species, e.g. $[Al_{13}(OH)_{30}(OH_2)_{18}]^{9+}$ designated Al_{13}, can develop at high Al concentrations, but they have not been detected in soil (Kinraide, 1991). In addition, the activities of monomeric Al species decrease in the presence of various organic anions such as citrate, malate, malonate, oxalate and quinate, and inorganic anions including phosphate, sulphate and fluoride, which bind them, forming insoluble complexes (Jones, 1998).

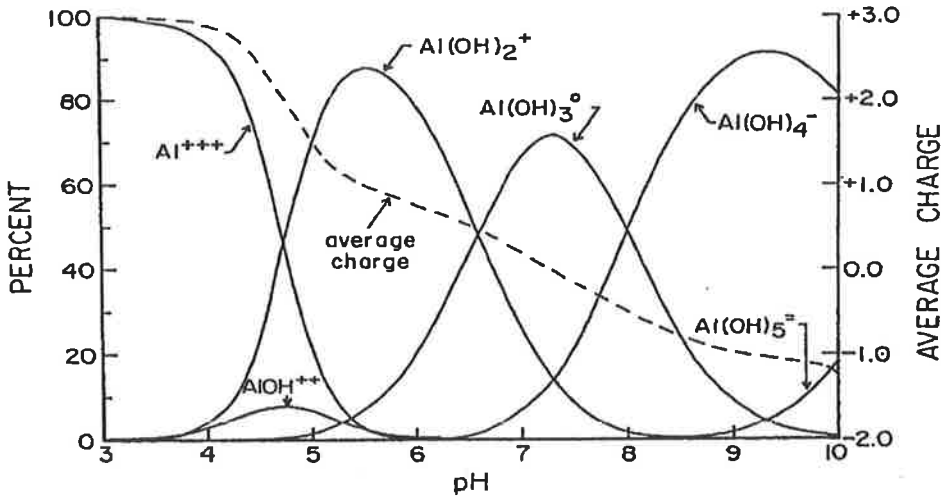

Figure 1.1 Relative distributions and average charge of soluble Al^{3+} and monomeric hydroxy species of aluminium as a function of solution pH (after Marion *et al.*, 1976)

1.2.1.1 Toxic species of aluminium

Aluminium is not considered an essential plant nutrient, and its presence in soil in high quantities is not a problem for plant growth particularly in neutral soils because it exists mostly as non-reactive forms (insoluble- organic or inorganic complexes), which are not harmful to living organisms. The element becomes detrimental to plants and soil microorganisms primarily under acidic conditions, since its concentration in solution increases with increasing soil acidity. At or below pH 5.5 soluble Al ions may achieve levels that are biologically toxic (Taylor, 1988). Products of Al hydrolysis all have the potential to trigger an Al stress response, but which species are more toxic to plants, whether the simplest Al^{3+}, hydroxy species of $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ (Alva *et al.*, 1986b) or the polymeric species Al_{13} (Kinraide and Parker, 1989), remains controversial. In addition to pH, other factors including soil organic matter and soil mineral composition can influence the activities of Al species and their toxicity to plants (Wolt, 1994; Robert, 1995). This complexity makes it very difficult to determine Al toxicity precisely under field soil conditions. Overall, the speciation of Al could be an important parameter of Al toxicity, but the toxicity of different Al species in soil solution has not been studied successfully (Wright, 1989). In consequence, many investigations use the extractable or soluble Al ions, referring to the collective concentration of monomeric Al ions, as the preferred predictor of Al toxicity in soil regardless of the species (Close and Powell, 1989; Wright, 1989; Wolt, 1994).

1.2.1.2 Phytotoxity of aluminium

Aluminium toxicity is a complex disorder expressed in various symptoms. The initial and most obvious symptom that identifies plants suffering Al toxicity appears on roots. The presence of Al in micro-molar concentrations in nutrient solution can inhibit root growth (Massot *et al.*, 1999). Excess Al restricts the elongation of the main root axis, the development of lateral roots and the formation of root hairs. As a result, roots become shortened and stubby with brown, occasionally necrotic thickened tips, and lacking root hairs (Care, 1995). Al-injured roots appear coralloid, with lack of fine branching. Such root systems cannot function effectively in the absorption of nutrients and water.

On shoots, Al toxicity symptoms appear as abnormalities in size, form and colour of leaves, leaf veins and stems. These foliar symptoms are often associated with those of P, Ca or Fe deficiencies (Foy, 1992). Commonly, seedlings are more susceptible to Al toxicity than older plants. In cases of severe damage, plants become stunted and suffer loss of yield (Baligar *et al.*, 1995).

Excessive Al in the growth medium affects several physiological and biochemical processes in plants. Mechanisms of Al phytotoxicity have been reviewed comprehensively by Taylor (1988) and other workers (Foy 1992; Fageria *et al*, 1988). In short, mechanisms include (1) replacement of Ca^{2+} or binding to free carboxyl groups of pectin, decreasing cell wall elasticity and so root elongation; (2) disorder of the integrity and permeability of plasma

membranes by binding to P on phospholipids or blocking Ca^{2+}-channels; (3) replacement of Ca^{2+} and Mg^{2+} in cytoskeletons that increases the rigidity of the skeleton; and (4) fixation of P in DNA, causing the double helix of DNA to become inflexible and the activity of the template depressed, resulting in disruption of nucleus functioning. Moreover, in soil, excess Al fixes P to form an Al-phosphate complex that reduces the solubility and thereby availability of P to plants. Excess Al can also hinder the uptake of some mineral nutrients, particularly Ca, Mg, K and Fe.

1.2.1.3 Plant tolerance to aluminium toxicity

Plant species exhibit different degrees of Al tolerance. Most are sensitive, but some are very tolerant. Plants may tolerate Al stress differently, depending on their capacity to tolerate higher concentration of Al in their tissues and their ability to exclude Al ions from the root apices (Foy, 1988). Inside the plant, there may be a specific regulation resulting in the detoxification of Al, but this remains little known. It has been proposed that mechanisms by which Al is detoxified internally may include formation of Al-organic anion complexes, and intracellular compartmentalization of Al to reduce its activity in cytosol. Recently there have been reports showing the presence of complexes of Al-citrate in *Hydrangea macrophylla* (Ma *et al.*, 1997a), and of Al-oxalate in *Melastoma malabathricum* (Watanabe *et al.*, 1998) and *Fagopyrum esculentum* leaves (Ma and Hiradate, 2000). The accumulation of Al in insensitive sites, such as epidermal cells and vacuoles was also found in Melastoma (Watanabe *et al.*, 1998). The mechanisms of exclusion

of Al from the root apices have also been demonstrated. Exudation of organic acids from Al-tolerant plant roots, as citrate (Miyasaka *et al.*, 1991), malate (Delhaize *et al.*, 1993) and oxalate (Zheng *et al.*, 1998), has been suggested as Al-chelators that precipitate non-toxic complexes at the root surface and thereby prevent Al penetration into the root cells. In addition, some workers believe that another mechanism of Al exclusion is increased rhizosphere pH, which is likely to decrease Al^{3+} solubility, and thereby reduce its chemical activity and toxicity (Degenhardt *et al.*, 1998; Bagayoko *et al.*, 2000). Furthermore, another important aspect of plant tolerance to Al is the nutritional status of the plant itself. The acquisition of some essential nutrients in sufficient quantities, mainly P, Ca, Mg and Fe, can increase plant tolerance to Al stress (Foy, 1992). Recent studies demonstrate that Calcium in soybean (Sanzonowicz *et al.*, 1998); Boron in mung bean (Yang and Zhang, 1998) and Silicon in maize (Ma *et al.*, 1997b) can contribute to reducing the deleterious effect of Al but the mechanisms are not entirely known.

Plant species may show different responses to excess Al when grown in solution and in soil cultures (Parker, 1995). The differences might be related to variation in the components comprising the culture medium. The contents of clay and organic matter (Edmeades *et al.*, 1995), and organic acids released by roots can be of importance in controlling the activities and hence the toxicity of Al species in soil (Jones, 1998) but not in solution cultures (Jorge and Arruda, 1997). In addition, the activity of microorganisms in soils, particularly those associating with plant roots, such as that of AM

fungi, can also be important in alleviation of the toxic effects of Al on plants (Rufyikiri *et al.*, 2000). This matter, particularly the contribution of AM fungi to plant growth, including under Al stress conditions, is discussed in more detail in the following section.

1.2.2 Arbuscular mycorrhizas

The term 'mycorrhizas' commonly refers to mutualistic symbioses between specific soil fungi and roots of higher plants, which benefit the two partners. The plants supply the fungi with organic carbon, and the fungi transport inorganic nutrients to the plants. To date, types of mycorrhizas have been recognized based on their morphological characteristics, including arbuscular mycorrhizas, ectomycorrhizas, ectendomycorrhizas, arbutoid, monotropoid, ericoid and orchid mycorrhizas (*see* Smith and Read, 1997). Arbuscular mycorrhizas (AM), which are the focus of this study, are the most widespread type in terms of geographical distribution and the number of plant species involved. AM fungi can be found in both natural and agricultural soils as a natural association involving about 80% of land plant species (Smith and Gianinazzi-Pearson, 1988). This type is established by soil fungi belonging to the order Glomales of Zygomycotina, in the genera of *Glomus, Sclerocystis, Entrophospora, Acaulospora, Gigaspora* and *Scutellospora* (Morton and Benny, 1990). The fungi are recognized as obligately dependent on living roots for organic carbon, and lack saprophytic ability (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999).

AM symbiosis is primarily characterized by specific structures of the fungus, inside and outside roots. Internal fungal structures differ with the pattern of root colonization and there are distinct *Arum* and *Paris* types (Smith and Smith, 1997). *Arum* type colonization is characterized mainly by internal hyphae that grow extensively between cortical cells, and arbuscules within the cells. An arbuscule, the little tree-like structure, is established by intercellular hyphae within a cortical cell 2-3 days after root penetration (Mosse, 1981). Arbuscules are highly branched, intracellular structures, which invaginate and are surrounded by the extended plasmalemma of the host cell. Although there is no direct contact between the fungal organ and cell cytoplasm (Bonfante-Fasolo, 1984), the structure provides a large surface area for the two partners, and the arbuscule is thought to be the site of nutrient transfer (Smith and Smith, 1990). The life span of arbuscules is short, estimated at 10-12 days in maize (Toth and Miller, 1984) and 6-8 days in leek (Dickson, 1999). Another internal structure formed by AM fungi except *Gigaspora* and *Scutellospora* is the vesicle. This is a terminal or intercalary swelling of internal hyphae; spherical, oval or lobed in shape with variation in sizes. This is typically formed after arbuscules and the number increases as plants mature. Vesicles contain lipid droplets and a number of nuclei so they may serve as endophytic storage organs, and function as fungal spores after the host plant dies (Biermann and Linderman, 1983). In *Paris* type colonization, AM internal structures are mainly intracellular, with coiled hyphae developing intensively and spreading from one and another cortical cells, with small arbuscules originating from the coils (Smith and Read,

1997). Detailed internal structure of the *Paris* type is less studied than that of the *Arum* type (Smith and Smith, 1997).

The external part of the AM system is the extraradical mycelium, i.e. external hyphae growing out into the bulk soil as the extension of internal hyphae. External hyphae are multinucleate, coenocytic and dimorphic, and consist of the main coarse hyphae and clumps of branched fine hyphae. The coarse hyphae have thick walls, diameters >20 μm, distinct angular projection, and the capability of reinitiating colonization and producing spores. The fine hyphae are considered as absorbing hyphae having thin walls and diameter 2-10 μm (Sylvia, 1992). The hyphae can extend up to 10-15 cm from a root surface and develop a network in the soil (Li *et al.*, 1991a; Jakobsen *et al.*, 1992a). Total length of the hyphae produced by AM fungal species differs extensively, ranging from 1 to 50 m g^{-1} soil (Sylvia, 1992) with most values 5 to 15 m g^{-1} soil (Jakobsen *et al.*, 1992b). Other external structures, e.g. auxiliary cells, are formed particularly by species of *Gigaspora* and *Scutellospora*, and asexual spores (chlamydospores) are formed in the external hyphae (see Smith and Read, 1997). Figure 1.2 illustrates the structure of AM based on *Arum* type colonization in particular.

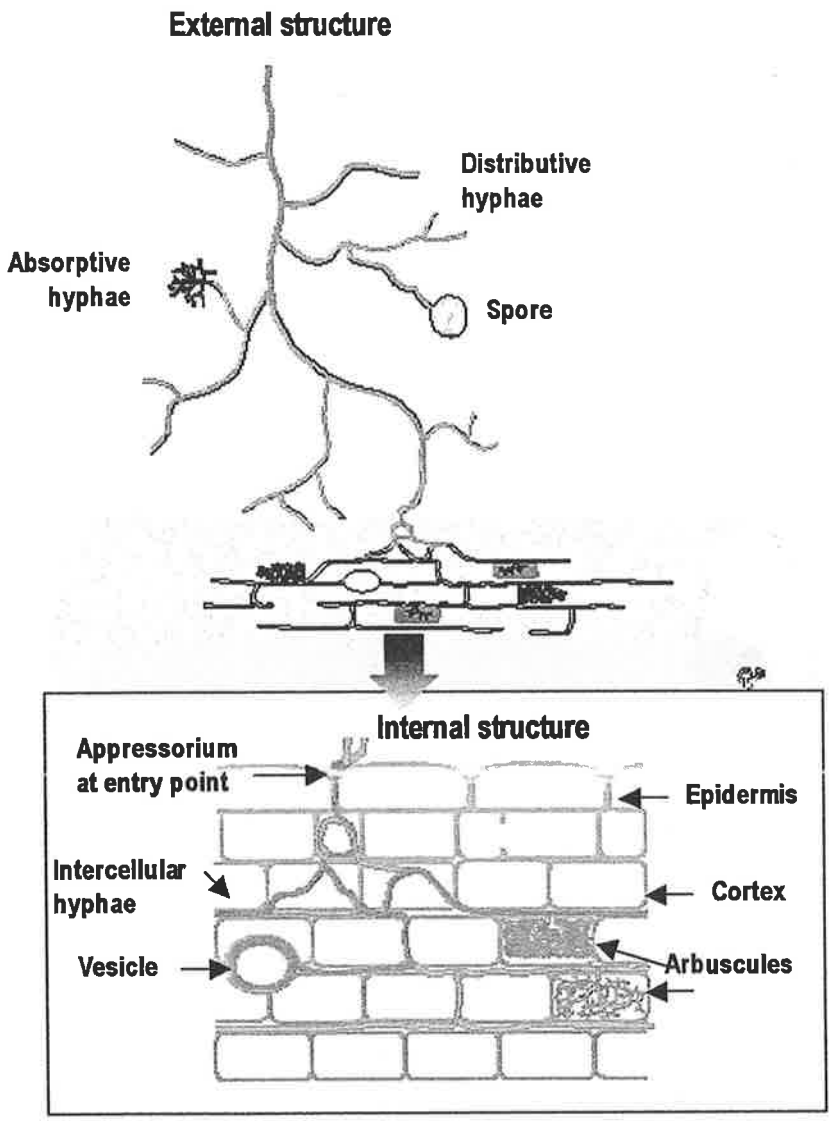

Figure 1.2 Schematic of external and internal structures of AM symbiotic system for the *Arum* type (after Brundrett *et al.*, 1996)

Mycorrhiza formation is a complex process consisting of a sequence of stages (*see* Fig. 1.3) that can be represented by events including spore germination and pre-symbiotic hyphal growth (2), hyphal branching in the presence of host roots, appressorium formation (3), root penetration and colonization, arbuscule development, external hyphal growth (4), and spore production in the soil (5) that may be followed by spore dormancy (1) (Giovannetti, 2000). It is believed that every stage involves a signaling-recognition process between the two partners (Harrison, 1999).

Root colonization can be initiated by hyphae growing from germinating spores or fragmented old colonized roots and/or external hyphae from neighboring colonized roots (Smith and Read, 1997). The growing hyphae can enter the root through root hairs or penetrate the epidermis directly via an appressorium or frequently between two epidermal cells, and then grow into the root cortex intercellularly through interconnected spaces and develop arbuscules (in the *Arum* type). In the case of roots lacking interconnected spaces, the hyphae may spread from cell to cell producing hyphal coils in the cortical cells (in the *Paris* type). In the subsequent stage, the fungus develops external hyphae that extend outside of the root, followed by producing spores in soil (Giovannetti, 2000).

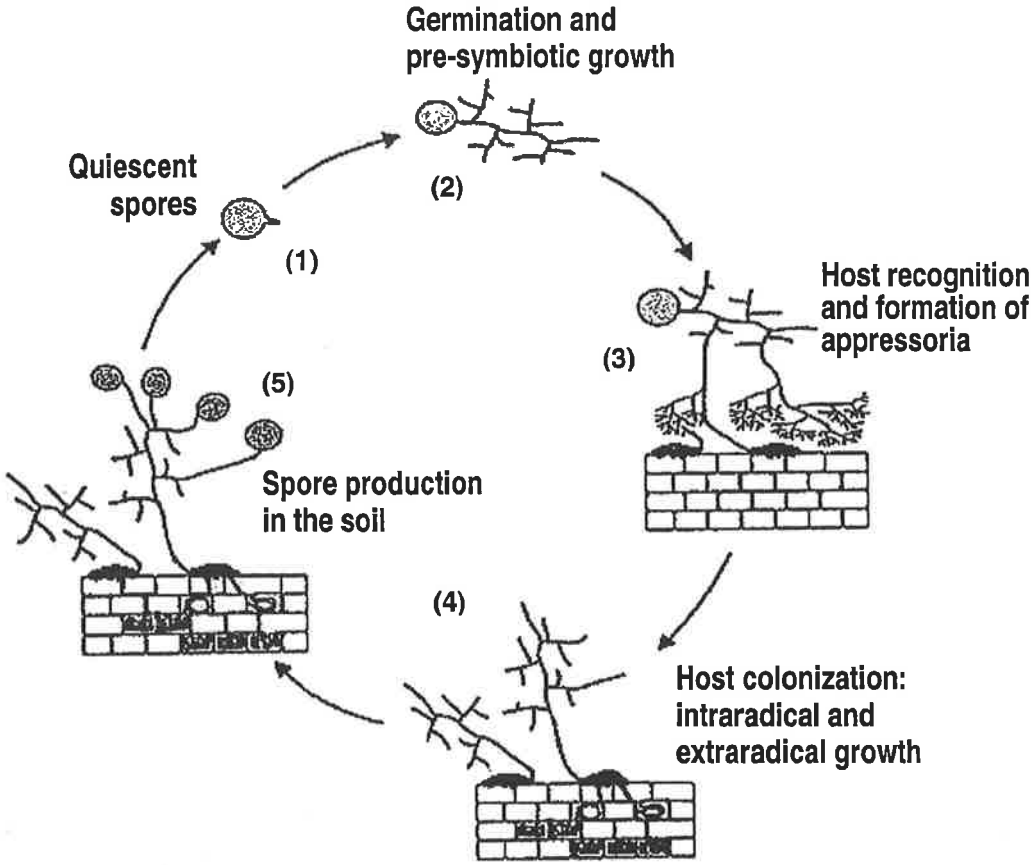

Figure 1.3 Schematic of the life cycles of arbuscular mycorrhizal fungi in relation to root colonization (after Giovannetti, 2000)

The formation process, however, may be impaired if the fungus fails to adapt to environmental conditions. Environmental soil factors, such as soil temperature, moisture (Daniels and Trappe, 1980), pH (Green *at al.*, 1976; Siqueira *et al.*, 1984), P availability (de Miranda and Harris, 1994), Al concentration (Bartolome-Esteban and Schenck, 1994), and microorganism activities (Azcón-Aguilar *et al.*, 1986) can influence the pre-symbiotic growth of the fungus, and so their potential to colonize plant roots. There is no

evidence that spore germination is affected by the presence of plant roots, but root exudates may stimulate the extension and branching of hyphae (Gianinazzi-Pearson *et al.*, 1989; Nagahashi and Douds, 2000). At later stages after the fungus has contacted roots, the process is more regulated by plant response, as indicated by the phenomena of abortive appressoria or fewer entry points on the root surface. Plant physiology in terms of nutritional status at that time is important to the regulation. Amijee *et al.* (1989) clearly demonstrated decreases in quantity of entry points, development of internal hyphae and length of root colonized in plants supplied with high P. Similarly, reduction in mycorrhiza formation was also observed when available P in soil was extremely low (Abbott *et al.*, 1984; Koide and Li, 1990). These findings generally suggest that despite the compatibility of the two partners, environmental factors may control the formation and accordingly the functioning of the symbiosis by affecting the plant and the fungus in different ways and at different phases of the colonization process.

1.2.2.1 AM function in plant growth

Beneficial effects of AM fungi on various crop species have been reported widely. Growth of mycorrhizal plants is often better than that of other plants without mycorrhizas, particularly under the stressful conditions of infertile soils. The growth improvement of mycorrhizal plants generally results from increased nutrient uptake. Mycorrhizal plants are more effective than non-mycorrhizal plants in P acquisition. Phosphate uptake efficiency in

mycorrhizal roots can be several times higher than in non-mycorrhizal roots (Nurlaeny *et al.*, 1996; Borie and Rubio, 1999). Other nutrients such as Ca, Mg, K, S, Fe, Zn, Cu, Mn and N are also increased in varying quantities by mycorrhizas (e.g. Raju *et al.*, 1988; Clark and Zeto, 2000). These increases are usually attributed to the contribution made by the external fungal hyphae.

External fungal hyphae are an essential part in the symbiosis, primarily acting as a bridge between plant and soil. They not only increase plant access to nutrients in soil by effectively enlarging the absorbing area of the root system and the soil volume exploited, but may also accelerate the acquisition process by reducing diffusion distances through the soil (Li *et al.*, 1991a; Jakobsen *et al.*, 1992a, b). Transport of nutrients, especially immobile elements such as P, Zn and Cu, through the hyphae from distant points beyond the root depletion zones, has been documented (e.g. George, 2000). In addition, by acidifying the surrounding soil (Li *et al.*, 1991b) or by releasing alkaline-phosphatases, the hyphae are able to exploit nutrients from sources unavailable for non-mycorrhizal roots, such as organic phosphates (Joner, 1994) or Ca-bound phosphates (Ness and Vlek, 2000).

Other benefits produced by AM fungi for host plants are also well known. These include increased plant tolerance to drought. Bethlenfalvay *et al.* (1988) reported that mycorrhizal roots were able to take up water at a lower soil water potential than non-mycorrhizal roots. Other studies found that under drought stress, mycorrhizal plants had lower resistance to water transport, so they had higher plant turgor, leaf water potential, stomatal conductance and transpiration rate compared to plants without mycorrhiza

(Safir *et al.*, 1972; Levy and Krikun, 1980; Augé *et al.*, 1986; Ebel *et al.*, 1997). AM symbiosis may also increase plant tolerance to infection by soil-borne pathogens, particularly when symbiosis is established before pathogen infection. Increased tolerance to root diseases is often attributed to increased nutrient uptake, but competition for infection sites between AM fungi and pathogens could be the main cause of reduction in root disease on mycorrhizal plants (Cordier *et al.*, 1996; Norman *et al.*, 1996). In some cases, however, reduction in mycorrhizal colonization by pathogens has also been reported (Dehne, 1982; Zambolim and Schenck, 1983). Moreover, mycorrhiza formation may protect plants from heavy metal toxicity. Although the mechanism is still unclear, regulation of the metal uptake by the fungi has been proposed. Koslowsky and Boerner (1989) showed that mycorrhizal switchgrass consistently contained less Al than non-mycorrhizal plants, though Al concentrations in growth media were increased. Heggo *et al.* (1990) found that compared with those of control plants without mycorrhizas, the concentrations of Zn, Cd and Mn in shoots of mycorrhizal soybean plants were lower in soil with high availabilities of these metals but higher when these metal availabilities in soil were low. On the other hand, AM fungi may induce phytotoxicity when taking up toxic metals, such as cadmium (Cd) or nickel (Ni), in sufficient quantity (Guo *et al.*, 1996; Joner and Leyval, 1997). Even though AM fungi to some extent can mitigate the toxicity of metals, such as Al, Zn, Cu and Cd, exposure to these metals at high concentrations can suppress the fungi themselves (Gildon and Tinker, 1981, 1983).

The function of AM is dependent on the dynamic interaction between the plant and the fungus, and their responses to environmental conditions (Sylvia and Williams, 1992). AM fungi themselves, however, show great differences in infectivity and effectiveness (referring to fungal ability to colonize roots and to improve plant growth or nutrient uptake respectively) (Abbott and Robson, 1981a; Krishna *et al.,* 1985a). In this respect, the infectivity of fungal species can be attributed to their inoculum potentials in soils (Abbott and Robson, 1981b), their inherent ability to adapt to soil conditions different from which they were isolated (Wilson, 1988), especially at the early stages of their growth (*see* section 1.2.2), and their capacity to interact with the plant hosts for development of colonization (Pinior *et al.*, 1999). Differences in effectiveness amongst species may be related to differences in the rapidity and the extent of colonization (Abbott and Robson, 1981b), the development and distribution of external hyphae in soils (Li *et al.*, 1991a: Jakobsen *et al.*, 1992a) and the capacity of hyphae to take up and transport nutrients from soil to roots (Abbott and Robson, 1982; Johansen *et al.*, 1993). Additionally, the ratio of internal and external mycelium, the number of connecting hyphae and the life span of the hyphae also influence the fungal effectiveness (Mosse, 1981).

As mentioned above, most terrestrial plant species can form AM symbioses, but there are considerable variations in root colonization between plant species or, even cultivars within a species occur (Plenchette *et al.*, 1983; Krishna *et al.*, 1985b; Baon *et al.*, 1993). Notwithstanding root morphology that is genetically inherent, the plant nutrition status induced by

external factors becomes the major regulator of the symbiosis. There is clear evidence showing that the extent of fungal colonization is often negatively correlated with the P status of the plant. Depressed root colonization and/or external fungal growth under high P has been observed for several plant and fungal species (Amijee *et al.* 1989; Thomson *et al.*, 1991; de Miranda *et al.,* 1989; Thingstrup *et al.*, 2000). Depression of AM function has also been observed when plants are grown in media with extremely low P (Abbott *et al.*, 1984; Koslowsky and Boerner, 1989; Koide and Li, 1990). In addition to soil P, other environmental factors can also influence the infectivity and effectiveness of AM fungi as already mentioned above (*see* Section 1.2.2; also Hayman, 1982; Sylvia and Williams, 1992: Smith and Read, 1997). These effects indicate that AM symbiosis can develop and function well when the plant and the fungus are compatible, and the environment is conducive. Nevertheless, uncertainty remains regarding the conditions of the environment that may optimize symbiotic functioning, especially in acid soils in which a set of factors exist which limit plant growth. It is, therefore, important to determine further ecological aspects of AM symbiosis under acidic soil conditions.

1.2.2.2 AM function at low pH

In acidic soil conditions (pH < 5.0) AM fungi are of considerable importance for plant growth, since plants often grow poorly because of loss of root function (Care, 1995; *see* Section 1.2.1.2). The promotional effects of AM

fungi on the growth and mineral uptake of several field crops on acid soils have been reported recently. These crops include sorghum (Medeiros *et al.*, 1994), maize (Clark and Zeto, 1996a; Nurlaeny *et al.*, 1996), soybean (Nurlaeny *et al.*, 1996), and barley (Mendoza and Borie, 1998; Borie and Rubio, 1999). These effects, however, vary with fungal species, crop species or cultivars and with soil pH. Medeiros *et al.* (1994) found that in sand culture (pH_{H2O} 4.8), the growth and mineral uptake of sorghum were significantly increased by *G. etunicatum*, but not by *G. intraradices*. On the other hand, Clark and Zeto (1996a) observed no differences amongst *G. intraradices, G. etunicatum* and *G. diaphanum* in stimulating growth and the uptake of P and Ca of maize grown on two acid soils (pH_{Ca} 4.2 and 4.6), although *G. intraradices* was more effective than the others for nutrients other than P and Ca. In contrast, Nurlaeny *et al.* (1996) found that *G. intraradices* contributed differently to the growth and P uptake of maize and soybean in two tropical acid soils, an Oxisol (pH_{Ca} 4.5) and an Ultisol (pH_{Ca} 4.0). In the Oxisol, the contribution increased when the soil pH was increased from 4.7 to 6.4, while the greatest contribution in the Ultisol was at pH 5.6.

Mycorrhizal colonization in acid soils also seems to vary with fungal species. Clark and Zeto (1996b) found no effects of low pH on the colonization by *G. intraradices, G. etunicatum* and *G. diaphanum* of maize roots, but increased soil pH increased colonization. In contrast, increased soil pH significantly decreased the root colonization by *G. intraradices* on soybean and maize (Nurlaeny *et al.,* 1996) and by *G. etunicatum* on an Al-sensitive barley cultivar (Borie and Rubio, 1999). Furthermore, the responses

of AM fungi to low soil pH varied with host plants. Root colonization by *G. macrocarpum* on *Guizotia abyssinica* was inhibited at pH 4.3 (Skipper and Smith, 1979), but not on *Tagetes minuta* (Graw, 1979). In addition, root colonization by *G. etunicatum* was different on two cultivars of barley differing in sensitivity to low pH when grown in sand culture at pH 4.8 (Mendoza and Borie, 1998).

Other reports showed that low pH restricts distribution and spore abundance of fungal species in the field (Porter *et al.*, 1987a), and inhibits spore germination and hyphal growth from germinated spores (Green *et al.*, 1976: Siqueira *et al.*, 1984). Thus it is likely that reduced mycorrhizal colonization at low pH resulted from inhibition of the early growth of the fungus. However, it is not clear which factor under acidic conditions is responsible for this inhibition. It is proposed that in acid soils low pH *per se* in terms of high H^+ ion activities (Porter *et al.*, 1987b), or high solubility of Al (Raju *et al.*, 1988: Borie and Rubio, 1999), accompanied by P deficiency (Abbott and Robson, 1985), becomes the major factor depressing AM fungal growth and functions, but due to their confounding effects they have never been demonstrated separately. Further work, which discriminates between the effects of Al and low pH, is needed. In the following section the importance of Al as the major factor affecting the mycorrhizal functioning in acid soils is discussed.

1.2.2.3 AM function under aluminium stress conditions

There have been many studies conducted on the responses of plants both to Al toxicity and to AM fungi under acidic conditions, but only few studies on the interactions between Al and AM fungi. Therefore, the effects of Al toxicity on the infectivity and effectiveness of AM fungi, and also of the ability of the fungus to alleviate the toxic effects of Al remain a large area of relative ignorance. The two aspects have, however, received a little attention (Robson and Abbott, 1989; Clark, 1997; Habte, 1999).

Some studies indicated that excessive Al in soil solution was the major inhibitor of AM fungi, though this varies in degree with species or isolates of the fungus. Bartolome-Esteban and Schenck (1994) showed that spore germination and hyphal growth of *Glomus* species, with the exception of *G. manihotis,* were completely inhibited by high concentrations of Al in soil, whereas those of most species of *Gigaspora* were not affected. Mendoza and Borie (1998) found that *G. etunicatum* made no contribution to an Al-sensitive barley cultivar grown on a sand culture containing 600 μM Al. White (1984 *cit.* Robson and Abbott, 1989) found *G. fasciculatum* unable to mitigate the negative effects of Al on clover even though its root colonization appeared unaffected.

So far, the mechanism by which Al inhibits the fungal growth and function remains unknown. Nevertheless, it is believed that excess Al may have direct effects on the fungus, mainly in the early stages of fungal growth, resulting in inhibited mycorrhiza formation. According to Robert (1995), the

toxicity of Al to fungi may result from the activities of Al ions that increase fungal cell wall rigidity, disruption of enzymatic and growth regulator activities, and fixation of P on the DNA double helix, reducing DNA replication. In addition, the excess Al may also reduce fungal growth via effects on the host plant. Plants suffering from Al toxicity often show P deficiency. This may in turn limit photosynthetic rates; accordingly not enough organic carbon will be produced and so will be unavailable for fungal growth. Koide and Li (1990) demonstrated that sunflower plants had a poorly developed mycorrhizal colonization when grown in sand culture supplied with very low P, and colonization increased as P increased. Koslowsky and Boerner (1989) suggested that a supply of P in sufficient amounts was needed to stimulate the ability of mycorrhiza to alleviate the toxic effect of Al on switchgrass. Therefore, under excess Al conditions inducing P deficiency in plants, restriction in the growth and function of AM fungi can occur due to the lack of organic carbon supply from the plants.

In spite of Al toxicity to the fungus, the ability of AM fungi to alleviate Al toxicity to plant growth has been demonstrated by some workers. Rufyikiri *et al.* (2000) found that the growth of mycorrhizal banana plants in a sand culture treated with 180 μM Al was better than that of plants without mycorrhiza. Medeiros *et al.* (1994) showed that *G. etunicatum* increased sorghum tolerance to toxic Al; no differences were observed in plant growth and P, Ca, Mg and Zn uptake of mycorrhizal plants grown in a sand culture containing Al in the range 0 - 105 μM Al. Mendoza and Borie (1998) found that the alleviation of Al toxicity by *G. etunicatum* varied with plant responses

to Al. In sand culture with 600 μM Al, the fungus significantly benefited an Al tolerant barley cultivar but not an Al sensitive one. Borie and Rubio (1999) found similar results by growing the two barley cultivars in soil culture with different Al concentrations. These findings clearly indicate that AM fungi can reduce Al phytotoxicity, although the extent depends greatly on the responses of the host plants themselves to Al stress and on their P status in particular. However, how mycorrhizas mitigate the negative effect of Al is unknown. Increase in mycorrhizal plant tolerance to Al may possibly result from improved plant nutritional status. The acquisition of P in sufficient quantity could help plants to escape from detrimental effects of Al by growing faster (Foy, 1992). It is also speculated that mycorrhizas could reduce Al toxicity by excretion of organic acids in the mycorrhizosphere, or by retaining Al in the fungal organs, such as vesicles, auxiliary cells or external hyphae (Koslowsky and Boerner, 1989).

1.2.3 Difficulties in Al-AM interaction studies

As mentioned above, many investigations of AM fungi have been made with acid soils, but there is no conclusive evidence showing which factors exert the strongest effect on AM fungi. In that regard, a few reports concerning the inhibition of AM function by Al remain mechanistically inconclusive in the sense that the toxic effect of Al was not separated from that of other stress factors related to soil acidity. The major problems in such studies mostly arise from the difficulty of fixing soluble Al ion levels in plant growth media,

particularly in soil because of the complexity of chemical reactions involving Al in soil solution. Therefore, because it is easier to establish soluble Al levels in solution, the toxicity of Al to plants is routinely studied using hydroponic techniques. However, this method is not really appropriate to mycorrhizal studies since AM fungi do not grow well under these conditions. Furthermore, the benefits of AM fungi to plant growth are related to their roles in soil. Some workers have employed semi-hydroponics in sand cultures to look at the effects of Al on AM function (Koslowsky and Boerner, 1989; Medeiros *et al.*, 1994; Mendoza and Borie, 1998; Rufyikiri *et al.*, 2000). This method was very promising and provided the possibility of investigating the effect of a single stress factor without affecting others. Nonetheless, to some extent the results would be different when soil or sand-soil mix is used, because sand culture is deficient in pH-buffering capacity that is important to maintain chemical conditions in soil (Edmeades *et al.*, 1995), and physically less conducive to external hyphal growth (E Drew, *personal communication*). Consequently, methods to set up soil with different soluble Al levels under particular soil conditions, such as pH and other soil chemical components, for studying AM fungi and Al interactions are a much needed development. Another difficulty in drawing conclusions about Al toxicity to AM fungi is related to the use of inappropriate experimental techniques; using the conventional pot it is virtually impossible to distinguish the direct effect of Al on the fungus from those of other acidity-related factors. Thus, development of an appropriate experimental procedure is necessary. The compartmental system introduced firstly by Schüepp (1987), and then developed in various models by other

workers (e.g. Li *et al.*, 1991a; Jakobsen *et al.*, 1992a, b; Vierheilig *et al.*, 1995), could be relevant for assessing the toxic effects of Al on AM fungi. Basically, the system provides space for external hyphae to grow alone, separated from colonized roots. Hence the effects of given treatments on the growth and function of external hyphae can be determined more easily.

1.2.4 Summary

The beneficial effects of AM fungi on plant growth have been well established. By extending and distributing their external hyphae into the soil beyond root depletion zones, these fungi can improve plant nutrient acquisition and resistance to some soil stress factors, particularly when grown in problem soils including infertile acid soils. However, plant benefits derived from AM may vary with plant genotypes and fungal species or isolates. A number of soil components, such as soil pH, nutrient availability, particularly P, and metal toxicity may also influence mycorrhizal functions. Little is known about environmental conditions under which the symbiosis functions optimally.

Al toxicity is definitely the most limiting factor for plants in acid soils. Excess Al inhibits the development of root systems, and limits the availability of mineral nutrients, especially P, causing problems in acquisition of nutrients and water in sufficient quantities. Furthermore, Al toxicity may also be expressed in reduction of the growth and activity of various microorganisms

in soils, particularly AM fungi, that have important contributions to plant nutrition.

Relatively few studies have been made of the interaction between AM fungi and Al toxicity. Some demonstrated that AM fungi could alleviate toxic effects of Al on plant growth, but others revealed that the growth and functions of the fungi are themselves depressed by Al. However, mechanisms by which AM fungi alleviate Al phytotoxicity or by which Al affects the growth and functions of the fungi are largely unknown. So, there is considerable opportunity to carry out studies in this area.

From the literature, there are some interesting findings that need to be clarified, particularly regarding the confounding effects of Al and low pH *per se* on AM fungi under acidic conditions, the stage in the fungal life mostly depressed by Al, and the ways in which Al affects the fungus whether directly or indirectly via host plants. Overall, these findings suggest that there is a requirement to develop a new approach in the study to make it possible to assess separately the effects of Al and low pH, and the effect of Al directly and indirectly via a host plant on AM fungi. Therefore, the establishment of growth media with specific conditions of pH and Al concentration and the use of appropriate pot systems to separate root and hyphal domains are essential.

1.3 Aims of Study

The overall aim of this study is to improve knowledge about the interaction between AM fungi and Al toxicity in acid soils, by focusing on the following questions:

1. Does low pH of itself inhibit the functioning of AM symbiosis?
2. Does Al toxicity of itself inhibit the functioning of AM symbiosis?
3. Does Al affect spore germination, and germ tube growth of the fungus?
4. Does Al directly affect external hyphal growth of the fungus or indirectly via the host plant?
5. Does plant P status influence external hyphal growth of the fungus under Al stress?

To answer the questions, this study began by establishing growth media having a range of independently varying pH and Al concentrations, and secondly choosing plant species moderately tolerant to low pH but rather sensitive to Al (Chapter 2) that offered the possibility of assessing separately the effects of low pH on plant responses to mycorrhizas (Chapter 3) from those of Al concentration (Chapter 4). Chapter 5 presents the investigation of spore germination and hyphal growth in agar plates and soil media with different Al concentrations. Furthermore, using compartmented pots, the effects of Al on the growth and functions of external hyphae were also assessed (Chapter 6). Chapter 7 presents an investigation of the effect of plant P status on the growth of external hyphae under Al stress. All these

results are then discussed to draw conclusions and suggestions for future work (Chapter 8).

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter describes materials and methods frequently used in the experiments. Variation in the materials or methods for specific experiment is described in the relevant chapters.

2.1 Growth medium

An important thrust of this study is the assessment as far as possible of the effect of aluminium separately from that of low pH. Accordingly, the establishment of plant growth media to meet these specific conditions required in this study was essential. For studies relating to the effects of low pH, the medium was adjusted to pH less than 5.3. Because of the impossibility of removing all soluble Al, its concentration in the medium was maintained at less than 2 ppm, which was not toxic to cowpea plants (*see* Section 2.2). In that way, the potential effect of Al could be minimized. For studies of the effects of Al toxicity, on the other hand, the growth media were set up at a certain pH but with different added soluble Al concentrations.

The growth medium was a mixture of sand and soil (90:10, w/w). The soil was an acidic podsolic soil of grey sandy loam, collected from Flaxley Farm, Adelaide Hills, 45 km of Southeast of Adelaide, South Australia. The soil sample was taken up from the topsoil of about 25 cm in depth. The soil

was air-dried, ground and sieved with a 5 mm screen, and then mixed completely with washed river sand (K40, Keough Sand Depot Pty. Ltd., South Australia). Some chemical properties of the soil are shown in Appendix 1.

In the preparation process, the medium was firstly fertilized with basal nutrients based on the composition of Ruakura solution for leguminous plants (Smith *et al.,* 1983). This was required to provide adequate nutrients for growth of experimental plants. The amounts of nutrients added were 59.4 NH_4-N, 178.2 NO_3-N, 36 P, 54 S, 214.2 K, 18.9 Mg, 114.3 Ca, 13.5 Na, 8.1 Cl, 2.7 Fe, 0.45 B, 0.45 Mn, 0.225 Zn, 0.036 Cu and 0.009 Mo mg kg^{-1} medium. Nutrients were mixed thoroughly and this medium was denoted M_O. To establish media having different pH values, M_O was treated by addition of different volumes of H_2SO_4, NaOH and purified water to produce final water content of 0.25 g g^{-1} soil, and mixing thoroughly at every addition. The media were then incubated for at least two weeks before being air-dried. These mixes were denoted $M_{4.6}$, $M_{4.9}$ and $M_{5.2}$ for media pH of 4.6, 4.9 and 5.2 respectively. Furthermore, to produce media containing different soluble Al concentrations, amounts of 0, 75, 150 or 300 μg Al g^{-1} medium as $Al_2(SO_4)_3$ solution was added to M_O and mixed thoroughly. Different volumes of NaOH solution were then added to adjust the pH to 4.7. Purified water was added to produce water content of 0.25 g g^{-1} soil. The soil was mixed completely, and incubated for at least two weeks before being air-dried. These mixes with the addition of 0, 75, 150 or 300 μg Al g^{-1} medium were denoted A_1, A_4, A_7 or A_{12}

respectively (the subscript refers to Al concentrations in the final established growth medium of 1.1, 4.1, 7.3 and 11.9 ppm).

Table 2.1 shows the solutions and volumes added in setting up the growth medium.

Table 2.1 Volumes of chemical solutions added to establish the growth media after fertilization with Ruakura solution, based on 1000 g medium

Medium	1 M H_2SO_4 (mL)	0.185 M $Al_2(SO_4)_3$ (mL)	1 M NaOH (mL)
M_O	0	0	0
$M_{5.2}$	0.19	0	0
$M_{4.9}$	0.75	0	0.19
$M_{4.6}$	1.13	0	0.56
A_1	1.13	0	0.75
A_4	0	7.5	2.88
A_7	0	15.0	10.63
A_{12}	0	30.0	24.25

The growth media were autoclaved for 1 hour at 121°C twice on each of two days prior to use to eliminate AM fungal propagules from the media.

Table 2.2 presents the composition of selected elements in the media.

Table 2.2 Selected chemical properties of the growth media used for experiments after adjustment and autoclaving

	Growth medium							
	M_O	$M_{5.2}$	$M_{4.9}$	$M_{4.6}$	A_1	A_4	A_7	A_{12}
pH_{H2O} (1:5)	5.32	5.16	4.89	4.68	4.73	4.69	4.74	4.70
EC_{H2O} (1:5) (dS/m)	0.31	0.28	0.39	0.31	0.29	0.35	0.51	0.73
[1]Elements (ppm)								
Ca	113	118	117	110	112	106	108	108
Mg	30	32	24	29	28	25	27	26
K	121	120	129	138	115	123	129	145
Na	30	29	29	33	29	88	237	527
S	42	41	42	44	59	133	213	450
Fe	2.1	2.1	2.8	2.8	2.8	2.7	2.5	2.6
Mn	0.39	0.39	0.40	0.39	0.38	0.44	0.42	0.43
Zn	0.52	0.53	0.49	0.54	0.56	0.50	0.54	0.54
[2]P-Bray 1	26	29	30	29	28	30	29	25
[3]Soluble-Al	0.4	0.4	0.8	1.1	1.1	4.1	7.3	11.9
[4]Total N (%)	0.12	0.11	0.11	0.11	0.11	0.10	0.11	0.10

[1]Elements were extracted in 0.01 M $CaCl_2$ (Houba *et al.*, 2000) and determined by ICP-AES; [2]Phosphate was extracted in 1 M NH_4F and determined colorimetrically with ammonium molybdate using a spectrophotometer (Bray-1 method) (Buurman *et al.*, 1996); [3]Al was extracted in 0.02 M $CaCl_2$ and determined colorimetrically with Chrome Azurol-S using a spectrophotometer (Close and Powell, 1989); [4]N was determined using Kjeldahl method (Buurman *et al.*, 1996).

2.2 Plant species

The plant species chosen for this study was cowpea (*Vigna unguiculata* L. Walp.). It is one of the important vegetable and protein source crops in tropical regions, mostly occupied by acid soils (Fageria *et al.*, 1997). The reasons for choosing this plant are that it is moderately tolerant to low pH but predisposed to Al toxicity (Howeler, 1991), and that it has a positive response to AM symbiosis (Yost and Fox, 1979; Thompson, 1991). These characteristics are needed to allow assessment of the effects of Al toxicity on mycorrhizal growth and functions on the plant separately from those of low pH. The cultivar of cowpea used in this study was Red Caloona, supplied by CSIRO Tropical Agriculture, Brisbane, Australia.

2.3 Preliminary growth experiment

A preliminary experiment to assess the cultivar's response to Al toxicity was carried out by comparing the growth of control plants in the untreated growth medium with pH 5.3 (M_O) to the growth of plants in the Al-treated growth media that contained 1.0, 2.6, 4.4 or 5.2 ppm soluble Al respectively. The method to set up the Al-treated growth media was as described in Section 2.1. Plants were grown singly in pots containing 700 g of growth medium and harvested 6 weeks after transplanting. There were 5 replications for every treatment. The plant weight was measured after drying at 70°C for 48 hours.

Fig. 2.1 presents data on plant growth in relation to soluble Al concentrations in growth media.

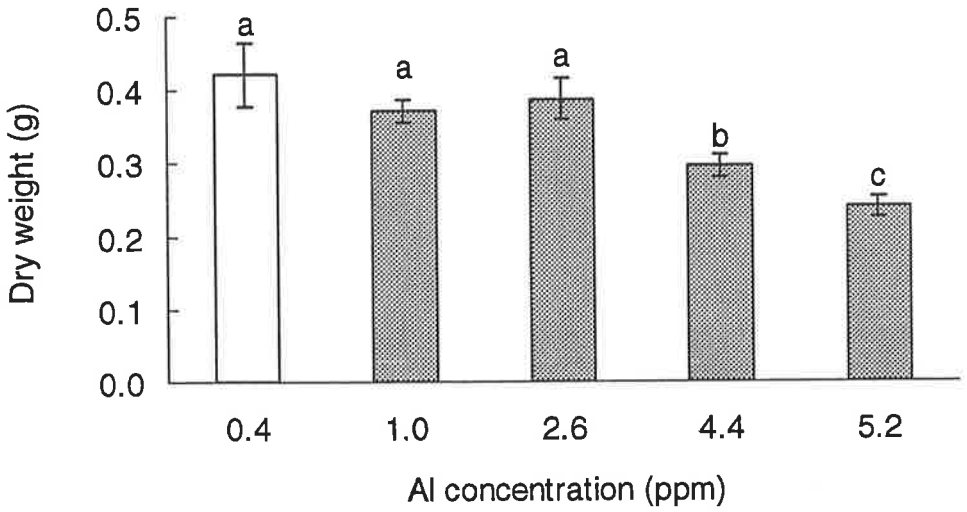

Figure 2.1 Growth of cowpea in relation to soluble Al concentrations in no Al-treated (Mo, open bar) and Al-treated soils (grey bars)

Bars represent means and standard errors of means (n=5); Bars marked with the same letters are not significantly different at $P \leq 0.0\,5$.

Overall, the plant growth decreased as Al concentrations increased. Roots of plants particularly at high Al were stunted and lacked lateral branches. Compared to control plants in Mo, a significant reduction in plant growth was observed only in growth media containing 4 ppm or higher soluble Al. This suggests that the cowpea cultivar has a limited tolerance to Al. Therefore, for further experiments the concentrations of Al in growth

media were set up in the range of toxic level of soluble Al concentrations to the plant.

2.4 AM fungal species

Gigaspora margarita Becker and Hall (originally obtained from INRA, Dijon, France prior to establishment of the BEG collection) was employed in all experiments, while *Glomus etunicatum* Becker and Gerdemann (UT316-9, INVAM collection) was only used in one experiment. Previous work showed that the two fungal species were effective in acidic conditions (Yawney *et al.*, 1982; Clark *et al.*, 1999; Borie and Rubio, 1999).

The isolates were propagated in pot cultures of subterranean clover (*Trifolium subterranean* L.) in a growth medium comprising a mixture (90:10, w/w) of sand and soil at pH 5.3 for 4 months. Another culture without mycorrhizas was prepared similarly to provide mycorrhiza-free inoculum for the experiments.

2.5 Plant growth and fungal inoculation

The seeds of cowpea were surface-sterilized with a 15% NaOCl solution for 15 minutes, washed twice with distilled water and then soaked in distilled water overnight. The seeds were then germinated on wetted filter paper in Petri dishes in the dark for three days before transplanting into experimental pots.

Plants were grown under glass-house conditions with an average air temperature range of 18-30°C (daytime). Most of the experiments were conducted around the summer season when full sunlight was available; no artificial light was used. Soil moisture was maintained by watering to weight at around field capacity (0.1 g g^{-1} soil) and pH in pots was maintained throughout experiments using reverse osmosis (RO) water adjusted with H_2SO_4 to the required pH. There was no leaching from the pots.

Plant inoculation was carried out using either spores or a mixed mycorrhizal inoculum (containing spores, external hyphae, fragmented roots and medium culture). Spores were extracted from pot cultures by wet sieving and sucrose centrifugation (*see* Brundrett *et al.*, 1996), and using a pipette a certain number of the spores in 2 mL water was then placed a few cm below the seeds at sowing times. Mixed inoculum was mixed thoroughly into the growth medium in a 10:90 (w/w) ratio prior to use. Spore filtrate (2 mL per pot) or mycorrhiza-free inoculum (a 10:90; w/w ratio) was given to control plants without mycorrhiza. The filtrate of spores was prepared by filtering suspension of extracted spores from pot cultures (containing about 5.10^3 spores in 25 mL water), using 30 µm screen mesh. No rhizobia were inoculated, to reduce the complication of biological interactions. The N requirement of plants was fulfilled from fertilization as shown in Section 2.1 (Smith *et al.*, 1983) regardless of the plants having nodules or not.

2.6 Pot systems

Different container systems were used, depending on experimental purposes. These included conventional pots with only one compartment where roots and external hyphae grew together and pots with two or three compartments separated by 30 μm screen mesh that provided a root-free zone for hyphae to grow separately from roots. The system of pots used in particular experiments is described in the relevant chapters.

2.7 Determination of plant biomass

At harvest, shoots were detached from roots, dried at 70°C for 48 hours and weighed to determine dry weight. Roots were carefully pulled out from pots and placed on a 2 mm sieve and washed under a stream of water. Washing was done repeatedly with care to remove adhering soil. This was important to avoid soil contamination when the roots were analyzed for element concentrations. Washed roots were then blotted dry and weighed to determine fresh weight. Samples were taken randomly for determination of mycorrhizal colonization. The remaining roots were dried at 70°C for 48 hours and weighed. Total root dry weight was determined from the fresh weight of the whole root system and the fresh/dry weight ratio of sub-samples.

Representative samples of growth media were collected from every pot or hyphal compartment using three soil cores (13 mm in diameter) prior to

harvest, which were mixed and air-dried. The samples were used for assessment of growth media pH and/or external hyphae.

2.8 Determination of root colonization

Root samples were cleared with 10% KOH, and stained with trypan blue in lacto-glycerol (a modification of the method of Phillips and Hayman (1970); omitting phenol from the reagents, and processed at room temperature). Stained roots were cut into 1 cm segments and spread randomly in a Petri dish to which a grid of 1 cm was attached. Total root length and the percentage of root length colonized by the AM fungi were assessed under a dissecting microscope with 40x magnification (Giovannetti and Mosse, 1980). The total number of intersections between roots and lines was tallied and later used to estimate the root length based on the formula $R = (11/14) \times N \times G$ (Tennant, 1975); where R = estimated root length in cm, N = number of intersections, and G = grid size (cm). Percentage of root colonized was calculated from counts of the number of intersections with presence of fungal structures and the total intersections.

2.9 Determination of external hyphal length

External hyphae were extracted from growth media and measured following the method of Jakobsen *et al.* (1992a) with some modification. A sub sample (approximately 3 g) of air dried growth medium was poured into a 30 µm sieve, rinsed gently with tap water to remove fine particles and transferred

into a blender which was then filled up to 250 mL with water. The soil suspension was blended at high speed for 15 s and poured quickly and quantitatively into a 250mL Erlenmeyer flask, closed with a stopper and shaken vigorously for 5 s by hand then left on the bench for 1 minute. Duplicate 5 mL aliquots were taken with a pipette inserted halfway into the solution and then each filtered through a 5µm Millipore filter (25 mm in diameter). Hyphae retained on the filters were stained with 0.05% trypan blue in lacto glycerol for 5 minutes, rinsed with purified water, and the filters were transferred to a microscope slide. This was carried out twice for every soil sample, so there were total of 4 filters per soil sample.

The length of hyphae was assessed by a gridline intersect method (Tennant, 1975), using the 10x10 eyepiece graticule under 200x magnification. The length of hyphae per g soil was calculated based on the number of intersects in 25 fields of view, the area of the filter, the volume of aliquot and the weight of the soil sample. There was no effort to distinguish between hyphae of mycorrhizal fungi and other fungi but the number of intersections counted for mycorrhizal samples was corrected for the mean of that of the equivalent control without mycorrhiza, prior to calculation of hyphal lengths of the fungus.

2.10 Determination of element uptake by plants

Dried shoots and roots were ground separately, and the plant materials (up to 250 mg) were placed into a 50 mL digestion tube, covered with 7 mL of a 6:1 mixture of nitric (70%) and perchloric (70%) acids, and allowed to stand overnight in a fume hood. The tubes were then placed into a Tecator^{R} digestion block with consecutive temperatures at 160°C and 180°C for 9 and 3 hours. After digestion, the digests were diluted to 25 mL with purified water.

The concentration of P in the digest was determined by the phospho-vanado-molybdate colorimetric method (Hanson, 1950) with a Shimadzu UV-1601 spectrophotometer. The concentration of other elements was determined using inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

Using data on concentration and dry weight, the contents of elements in shoots and roots were calculated; these allowed the total quantity of element uptake by plants to be estimated.

2.11 Determination of mycorrhizal growth responses

Plant responses to mycorrhizas expressed as the percentage change in total plant growth or mineral uptake, were calculated with the formula:

$$[\{Plant_{(M)} - Plant_{(NM)}\} / Plant_{(NM)}] \times 100$$

where $Plant_{(M)}$ = dry matter or mineral content of mycorrhizal plants,

$Plant_{(NM)}$ = dry matter or mineral uptake of plants without mycorrhiza.

The efficiency of the plant root system in absorbing mineral nutrients was determined as specific uptake of nutrient, which refers to the amount taken up per unit root length at the final harvest.

2.12 Soil analysis

Soil pH and electrical conductivity (EC) were measured in H_2O (1:5, w/v) using an Expandable Ion Analyzer EA 940 Orion Research pH meter and an ATIorion Model 170 conductivity meter respectively. Available P in soil was extracted using NH_4F-HCl (Bray-I method), and determined colorimetrically using stanno-ammonium-molybdate; organic carbon was determined using Walkley and Black method; and total N was digested followed wet oxidation of Kjeldahl method and determined with Auto-analyzer for ammonium (Buurman *et al.*, 1996). Soluble Al ions were extracted with 0.02 M $CaCl_2$ and their 'reactive' concentrations determined colorimetrically using Chrome Azurol-S (CAS) following the method of Close and Powell (1989); and other elements were extracted with 0.01 M $CaCl_2$ (Houba *et al.*, 2000) and determined by ICP-AES.

2.13 Data analysis

Data were analyzed statistically using analysis of variance (ANOVA) to examine the differences amongst treatments. The LSD test at $P \leq 0.05$ was then used to determine if significant differences amongst treatments existed. Correlation or regression analyses were applied where appropriate. Data, in

some cases, were transformed prior to analysis. For the statistical analyses the package of GenStats 5th Edition, release 4.2 was used.

Data presented in tables are the means of different numbers replications, as stated.

CHAPTER 3

MYCORRHIZAL COWPEA GROWTH AT LOW pH

3.1 Introduction

The importance of AM symbiosis in improving plant growth and nutrient uptake has been well documented (*see* Smith and Read, 1997; Clark and Zeto, 2000; George, 2000). This includes observations in acid soils in which various factors restrict plant growth (Yost and Fox, 1979; Raju *et al.*, 1988; Clark and Zeto, 1996b; Mendoza and Borie, 1998; Nurlaeny *et al.*, 1996). The beneficial effects of the symbiosis, however, vary greatly with the fungal and plant species, and with soil conditions (Nurlaeny *et al.*, 1996; Clark and Zeto, 1996a, b).

Although the effects of soil acidity on the function of AM fungi have been studied for a long time (Green *et al.*, 1976; Habte, 1999), the factor exerting the strongest effect on the fungi remains unspecified. Most studies have proposed that Al toxicity, accompanied by deficient P, is predominantly responsible for limiting AM symbiosis in acid soils (Raju *et al.*, 1988; Medeiros *et al.*, 1994; Yawney *et al.*, 1982; Nurlaeny *et al.*, 1996). The inhibitory effect of low pH *per se* in terms of high H^+ ion concentrations on AM fungal growth and function was only reported by Porter *et al.* (1987b), but this is questionable because no information was provided on the concentrations of Al in the soil used.

This chapter describes an experiment that was carried out to elucidate whether low pH *per se* can have a significant effect on the functioning of two AM fungi in the growth and nutrient uptake of cowpea.

3.2 Materials and methods

The experiment was a factorial experiment with three pH levels (4.6, 4.9 and 5.2 of growth media $M_{4.6}$, $M_{4.9}$ and $M_{5.2}$), two fungal species (*Gi. margarita* or *G. etunicatum*) and controls without AM fungal inoculum. It had a completely randomised design with four replications. Pots without plants or inoculation were included to check changes in pH of the growth media during the experiment. This experiment was conducted under glasshouse conditions from October to December 2000. The plant, fungal species and growth media used in this experiment are described in Chapter 2.

Pre-germinated seeds of cowpea were transplanted singly to pots (10 cm high and 9 cm diameter) containing 700 g of the growth medium. Plants were inoculated with 250 spores of the each fungus or, in the case of non-mycorrhizal plants, their filtrates. The pH and moisture contents in pots were maintained throughout the experiment at about field capacity by RO water adjusted using H_2SO_4 to pH 4.6, 4.9 or 5.2. There was no leaching from the pots.

Plants were harvested 6 weeks after transplanting. Shoot and root dry weights, the uptake of P and other selected elements and the percentages of root length colonized by the AM fungi were measured. Also, plant response

to mycorrhizal inoculation and nutrient uptake efficiency (the amount of element taken up per unit root length) were calculated.

Data were analyzed statistically using ANOVA. Differences were then examined based on LSD values at $P \leq 0.05$ as described in Chapter 2. A correlation analysis was used to look at the relationship between root colonization and mycorrhizal growth response.

3.3 Results

3.3.1 Growth media pH

With the aid of maintenance during the experiment, the pH of growth media increased only slightly. The greatest increases were observed in un-inoculated pots at pH 4.6 (0.19 pH units) and in those containing plants inoculated with *Gi. margarita* at pH 5.2 (0.25 pH units) (Table 3.1).

Table 3.1 Growth media pH measured before and after the experiment

Growth media		$M_{4.6}$	$M_{4.9}$	$M_{5.2}$
Before experiment		4.68	4.89	5.16
After experiment				
Unplanted pots		4.66	4.93	5.29
Planted pots	No mycorrhiza	4.87	4.91	5.28
	Gi. margarita	4.68	4.92	5.41
	G. etunicatum	4.66	4.98	5.21

3.3.2 Root colonization

Root colonization by the two fungi differed and was affected differently by pH (Fig. 3.1). Percentages of root length colonized by *G. etunicatum* were significantly lower than those for *Gi. margarita*. A decrease in pH from 5.2 to 4.6 depressed the root colonization by *G. etunicatum* but slightly increased that by *Gi. margarita*. No mycorrhizal colonization was observed in roots of plants in uninoculated pots.

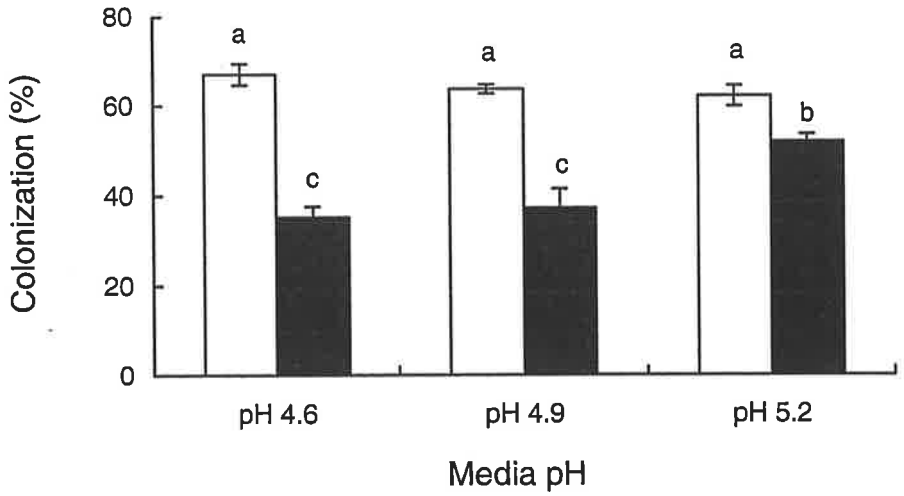

Figure 3.1 Root colonization of cowpea by *Gi. margarita* (□) and *G. etunicatum* (■) at different media pH.

Bars marked with the same letters are not significantly different at P≤0.05. Bars represent means and standard errors of means (n=4)

3.3.3 Plant growth

There was no interaction between pH of growth media and mycorrhizal inoculation in affecting plant growth, but the two factors independently had significant effects on both shoot and root dry weights, and on root length (Table 3.2). At pH 4.6 the growth of plants without mycorrhiza was significantly depressed. The plants showed nutrient deficiency symptoms, such as brown spot and/or necrosis on leaves; the lower leaves fell off prematurely, and the new leaves were smaller than the older ones. Also root development was poor. Shoot dry matter and root length were significantly higher at both pH 4.9 and 5.2 than at pH 4.6, but there was little difference

amongst root dry weights and shoot-root ratios in relation to the pH treatments.

Irrespective of pH, *Gi. margarita* increased both shoot and root weights of plants dramatically compared to both non-mycorrhizal plants and plants inoculated by *G. etunicatum*, but decreased shoot-root ratios (Table 3.2). No significant effect of *G. etunicatum* was observed in increasing plant growth (Table 3.2).

Table 3.2 Growth of mycorrhizal and non-mycorrhizal cowpea at different media pH

Media pH	Mycorrhizal inoculation	Dry weights of (g)			Shoot/root ratios	Root length (m)	MGR (%)
		Shoots	Roots	Plant			
4.6	No mycorrhiza	0.12^{c}	0.05^{b}	0.17^{c}	2.4^{ab}	1.4^{d}	0
	Gi. margarita	0.38^{a}	0.25^{a}	0.63^{a}	1.5^{c}	3.6^{c}	183
	G. etunicatum	0.21^{bc}	0.09^{b}	0.30^{bc}	2.3^{ab}	1.9^{cd}	45
4.9	No mycorrhiza	0.26^{b}	0.09^{b}	0.36^{b}	2.9^{a}	4.4^{b}	0
	Gi. margarita	0.45^{a}	0.25^{a}	0.70^{a}	1.8^{b}	7.6^{a}	95
	G. etunicatum	0.27^{b}	0.11^{b}	0.38^{b}	2.4^{ab}	5.1^{b}	7
5.2	No mycorrhiza	0.27^{b}	0.11^{b}	0.38^{b}	2.4^{ab}	4.1^{bc}	0
	Gi. margarita	0.47^{a}	0.23^{a}	0.70^{a}	2.0^{bc}	7.2^{a}	84
	G. etunicatum	0.29^{b}	0.11^{b}	0.40^{b}	2.6^{ab}	5.1^{b}	6

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$;

Mycorrhizal growth response (%MGR) = $[\{\text{Plant DW}_{(M)} - \text{Plant DW}_{(NM)}\} / \text{Plant DW}_{(NM)}] \times 100$

$\text{Plant DW}_{(M)}$: dry weight of a mycorrhizal plant;

$\text{Plant DW}_{(NM)}\}$: average of dry weight of non-mycorrhizal plants (n=4)

The growth response of plants to *Gi. margarita* appears to be closely associated with percentage of root length colonized, but this was not so for *G. etunicatum* (Fig. 3.2).

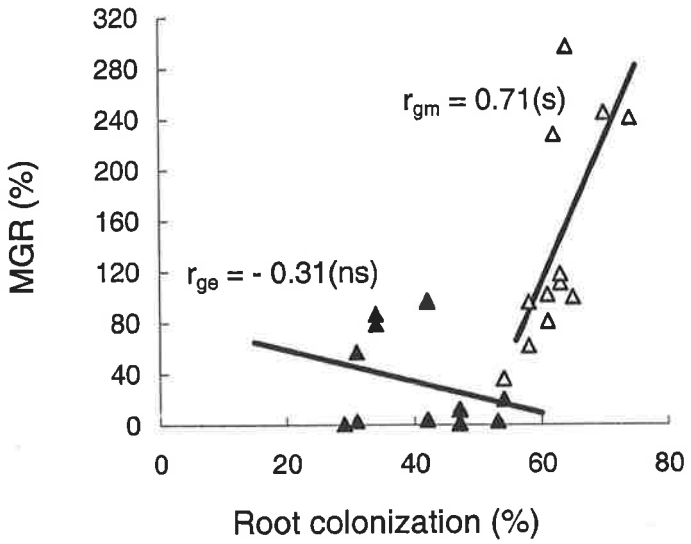

Figure 3.2 Relationship between mycorrhizal growth response (MGR) and root colonization by *Gi. margarita* (Δ) and *G. etunicatum* (▲)
s and ns: significant and non significant at P ≤ 0.05

3.3.4 Element concentrations in plant tissues

The effects of mycorrhizal inoculation on element concentrations in shoots and roots of cowpea plants in relation to growth media pH varied considerably between plants colonized by *Gi. margarita* and *G. etunicatum* and amongst elements, as shown in Tables 3.3 and 3.4.

For element concentration in shoots (Table 3.3), compared to non-mycorrhizal plants, *Gi. margarita* plants had higher P at pH 5.2, Mg at pH 4.6, S at pH 4.9 and 5.3, and Ca at all pH levels tested, but lower Mn at pH 4.9, Zn at pH 5.2, Al at pH 4.6 and 5.2, and Fe and Na at all pH levels. Compared to *G. etunicatum* plants, *Gi. margarita* plants had higher P and S at pH 5.2 but lower Ca, K and Fe at pH 4.6, Mg and Mn at pH 4.6 and 4.9, and Na at all pH levels tested. *G. etunicatum* plants show higher in S at pH 4.9, Mn at pH 4.6, and Ca and Mg at all pH levels, but lower Fe at pH 4.9 and Al at pH 4.6 and 5.2 compared to those in non-mycorrhizal plants.

For element concentrations in roots (Table 3.4), *Gi. margarita* plants had higher P at all pH levels, Mg at pH 5.2, and Na at pH 4.9 and 5.2, but lower S at pH 4.9 and K and Zn at pH 4.9 and 5.2 compared to those in non-mycorrhizal plants; and compared to *G. etunicatum* plants, *Gi. margarita* plants had higher P, Mg and Na at pH 5.2 but lower Ca and Zn at pH 4.6 and 4.9, K at pH 4.9 and 5.2 and S at pH 4.9. *G. etunicatum* plants had higher P and Zn at pH 4.9, K at pH 4.6, S and Na at pH 4.6 and 4.9, but lower Zn at pH 5.2 compared to those in non-mycorrhizal plants.

Table 3.3 Element concentrations in shoot tissues of mycorrhizal and non-mycorrhizal cowpea plants at different media pH

Media pH	Mycorrhizal inoculation	Element concentration									
		P (%)	Ca (%)	Mg (%)	K (%)	S (%)	Fe (mg/g)	Mn (mg/g)	Zn (mg/g)	Na (%)	Al (mg/g)
4.6	No mycorrhiza	0.12^{a}	3.00^{e}	0.92^{f}	4.77^{ab}	0.99^{a}	0.77^{a}	0.29^{d}	0.07^{ab}	0.43^{a}	0.17^{a}
	Gi. margarita	0.12^{ab}	3.40^{d}	1.04^{cde}	4.30^{bc}	0.99^{a}	0.26^{cd}	0.36^{cd}	0.07^{ab}	0.10^{c}	0.06^{b}
	G. etunicatum	0.13^{a}	3.83^{bc}	1.14^{ab}	5.27^{a}	0.93^{a}	0.57^{ab}	0.47^{ab}	0.08^{ab}	0.35^{ab}	0.05^{b}
4.9	No mycorrhiza	0.11^{b}	3.00^{e}	1.00^{ef}	4.00^{c}	0.84^{b}	0.57^{ab}	0.49^{a}	0.08^{ab}	0.38^{ab}	0.11^{ab}
	Gi. margarita	0.11^{ab}	3.57^{cd}	1.08^{bcde}	3.90^{c}	1.02^{a}	0.26^{cd}	0.40^{bc}	0.06^{b}	0.12^{c}	0.11^{ab}
	G. etunicatum	0.09^{b}	3.67^{cd}	1.18^{a}	4.18^{bc}	1.00^{a}	0.39^{cd}	0.50^{a}	0.07^{ab}	0.43^{a}	0.12^{ab}
5.2	No mycorrhiza	0.10^{b}	3.67^{cd}	1.02^{de}	3.68^{c}	0.72^{c}	0.47^{bc}	0.39^{bc}	0.09^{a}	0.31^{b}	0.16^{a}
	Gi. margarita	0.14^{a}	4.33^{a}	1.10^{abcd}	3.93^{c}	0.83^{b}	0.24^{d}	0.34^{cd}	0.06^{b}	0.08^{c}	0.06^{b}
	G. etunicatum	0.09^{b}	4.13^{ab}	1.11^{abc}	3.90^{c}	0.72^{c}	0.34^{cd}	0.43^{bc}	0.07^{ab}	0.33^{b}	0.06^{b}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$.

Table 3.4 Element concentrations in root tissues of mycorrhizal and non-mycorrhizal cowpea plants at different media pH

Media pH	Mycorrhizal inoculation	Element concentration									
		P (%)	Ca (%)	Mg (%)	K (%)	S (%)	Fe (mg/g)	Mn (mg/g)	Zn (mg/g)	Na (%)	Al (mg/g)
4.6	No mycorrhiza	0.11^{d}	0.79^{bc}	0.30^{d}	3.15^{d}	1.00^{e}	2.46^{a}	0.05^{a}	0.13^{def}	0.39^{d}	1.10^{ab}
	Gi. margarita	0.18^{abc}	0.64^{c}	0.39^{bcd}	3.53^{cd}	1.28^{de}	3.73^{ab}	0.04^{a}	0.11^{ef}	0.55^{bcd}	1.16^{ab}
	G. etunicatum	0.11^{cd}	0.88^{ab}	0.37^{cd}	4.70^{bc}	1.43^{cd}	4.10^{a}	0.04^{a}	0.16^{cd}	0.63^{bc}	1.35^{a}
4.9	No mycorrhiza	0.01^{d}	0.84^{ab}	0.39^{bcd}	7.05^{a}	2.05^{b}	2.17^{b}	0.04^{a}	0.14^{de}	0.48^{cd}	1.33^{a}
	Gi. margarita	0.19^{ab}	0.68^{bc}	0.50^{b}	3.35^{d}	1.35^{cde}	3.35^{ab}	0.04^{a}	0.09^{f}	0.82^{ab}	1.12^{ab}
	G. etunicatum	0.14^{bc}	0.91^{a}	0.47^{bc}	6.77^{a}	2.30^{a}	2.45^{ab}	0.04^{a}	0.17^{bc}	0.74^{ab}	0.91^{ab}
5.2	No mycorrhiza	0.12^{cd}	0.89^{a}	0.43^{bc}	6.83^{a}	1.69^{c}	2.95^{ab}	0.04^{a}	0.21^{a}	0.57^{bcd}	0.79^{b}
	Gi. margarita	0.23^{a}	0.89^{a}	0.64^{a}	5.17^{b}	1.56^{cd}	2.85^{ab}	0.05^{a}	0.17^{bc}	0.84^{a}	0.78^{b}
	G. etunicatum	0.13^{bc}	0.90^{a}	0.43^{b}	6.92^{a}	1.56^{cd}	2.71^{ab}	0.04^{a}	0.17^{bc}	0.56^{bcd}	1.04^{ab}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$.

3.3.5 Element uptake

Both pH and mycorrhizal inoculation affected element uptake by the plants, and significant interactions between the two factors were noticed for some elements, such as K, Fe, Zn and Al (Table 3.5). In general, the uptake of elements by non-mycorrhizal plants was not different at pH 5.2 and 4.9, but was reduced significantly at pH 4.6. The two AM fungi had different effects. Plants inoculated with *Gi. margarita* generally took up all the elements measured to a much greater extent than those inoculated with *G. etunicatum*, regardless of the pH. However, the quantities of these elements taken up by plants inoculated with *Gi. margarita* varied slightly with pH. The total uptake of Ca, Mg, S, Mn and Na at pH 4.6 was significantly lower than at pH 4.9, while there was no difference between uptake at pH 4.9 and 5.2, except for Ca and Na. The uptake of Ca increased as pH increased, but uptake of K and Zn did not change. In contrast, reduced uptake of Fe and Al was observed with a pH increase from 4.9 to 5.2. Compared to non-mycorrhizal plants, increases in total element uptake by plants inoculated with *G. etunicatum* were observed at pH 4.6 for all elements measured except Al, and at pH 4.9 for Ca only.

The effects of the two fungi in increasing nutrient uptake at low pH are summarized in Table 3.6. It appears that the increases attributable to mycorrhizal colonization were most pronounced at pH 4.6 for almost all of the elements measured. However, *Gi. margarita* was more effective than *G. etunicatum*. For instance, at pH 4.6 *Gi. margarita* increased P uptake by

about 350% compared to only 100% for *G. etunicatum.* A small increase of Ca, Mg, K and Mn uptake in plants colonized by both fungi was observed in going from pH 4.9 to 5.2. On the other hand, a negative response was also observed for the uptake of Al at pH 4.9, and of Fe and Zn at pH 5.2 with *G. etunicatum.*

Mycorrhizal colonization altered the efficiency of absorption of some elements (Table 3.7). There were increases at pH 4.6 for P, S and Al for plants inoculated with *Gi. margarita*, and for Ca, K, S and Mn for plants inoculated with *G. etunicatum.* At higher pH the element uptake efficiency was not different amongst plants with or without mycorrhizas, except that P uptake efficiency was higher in plants inoculated with *Gi. margarita* at pH 4.9 and 5.2, and lower in plants inoculated with *G. etunicatum* at pH 4.9 and 5.2. Moreover, it seems that the efficiency of the plant root system was more influenced by pH than by mycorrhizal inoculation. In this regard, the quantities of elements taken up per unit root length decreased slightly when pH decreased from 5.2 to 4.9, but then increased dramatically with a decrease in pH from 4.9 to 4.6.

Table 3.5 Total uptake of elements by mycorrhizal and non-mycorrhizal cowpea plants at different media pH

Media pH	Mycorrhizal inoculation	Element (mg plant^{-1})									
		P	Ca	Mg	K	S	Fe	Mn	Zn	Na	Al
4.6	No mycorrhiza	0.20^{d}	4.2^{f}	1.3^{d}	7.4^{c}	1.8^{e}	0.19^{d}	0.04^{d}	0.016^{d}	0.70^{d}	0.08^{c}
	Gi. margarita	0.88^{b}	14.9^{c}	4.9^{b}	25.2^{a}	6.9^{b}	0.99^{a}	0.15^{bc}	0.054^{ab}	1.70^{bc}	0.31^{a}
	G. etunicatum	0.39^{c}	9.9^{e}	3.0^{c}	17.0^{b}	3.6^{d}	0.54^{bc}	0.11^{c}	0.034^{c}	1.44^{c}	0.14^{bc}
4.9	No mycorrhiza	0.37^{c}	9.4^{e}	3.2^{c}	18.7^{b}	4.5^{cd}	0.35^{c}	0.14^{bc}	0.038^{c}	1.60^{bc}	0.17^{b}
	Gi. margarita	1.00^{ab}	18.5^{b}	6.4^{a}	27.1^{a}	8.3^{a}	0.99^{a}	0.20^{a}	0.053^{ab}	2.69^{a}	0.35^{a}
	G. etunicatum	0.38^{c}	11.3^{d}	3.8^{c}	19.0^{b}	5.2^{c}	0.38^{c}	0.14^{bc}	0.039^{c}	1.99^{bc}	0.13^{bc}
5.2	No mycorrhiza	0.40^{c}	10.9^{de}	3.2^{c}	17.3^{b}	3.8^{d}	0.44^{c}	0.11^{c}	0.046^{bc}	1.44^{c}	0.13^{bc}
	Gi. margarita	1.09^{a}	22.6^{a}	6.6^{a}	29.0^{a}	7.2^{ab}	0.66^{b}	0.17^{ab}	0.062^{a}	2.17^{b}	0.19^{b}
	G. etunicatum	0.40^{c}	14.0^{cd}	4.0^{c}	20.4^{b}	4.1^{d}	0.41^{c}	0.14^{bc}	0.041^{c}	1.70^{bc}	0.14^{bc}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$.

Table 3.6 Mycorrhizal element uptake responses at different media pH

Media pH	Mycorrhizal inoculation	Elements (% response)									
		P	Ca	Mg	K	S	Fe	Mn	Zn	Na	Al
4.6	*Gi. margarita*	336*	255*	285*	242*	292*	431*	308*	238*	142*	289*
	G. etunicatum	96*	136*	134*	131*	105*	186*	210*	108*	104*	80
4.9	*Gi. margarita*	172*	97*	96*	45*	85*	187*	38*	40*	68*	104*
	G. etunicatum	4	20*	17	2	17	8	0	4	25	(-22)
5.2	*Gi. margarita*	173*	108*	106*	67*	91*	49*	56*	34*	51*	45
	G. etunicatum	2	28	23	18	9	(-8)	25	(-10)	18	10

Mycorrhizal element uptake response (%) = $[\{Uptake_{(M)} - Uptake_{(NM)}\} / Uptake_{(NM)}] \times 100$
Values marked with * are based on values significantly different in comparisons between mycorrhizal (M) and non-mycorrhizal (NM) plants in Table 3.5.

Table 3.7 Element uptake per unit of root length by mycorrhizal and non-mycorrhizal cowpea plants at different media pH

Media pH	Mycorrhizal inoculation	Elements (µg cm^{-1})									
		P	Ca	Mg	K	S	Fe	Mn	Zn	Na	Al
4.6	No mycorrhiza	1.78^{b}	38^{bc}	12^{ab}	70^{b}	15^{bc}	2.2^{a}	0.38^{bc}	0.14^{ab}	6.8^{a}	0.67^{bc}
	Gi. margarita	2.47^{a}	45^{b}	16^{a}	75^{ab}	20^{a}	3.0^{a}	0.46^{ab}	0.16^{a}	5.2^{ab}	1.09^{a}
	G. etunicatum	2.13^{b}	55^{a}	17^{a}	93^{a}	20^{a}	2.9^{a}	0.63^{a}	0.18^{a}	7.9^{a}	0.85^{ab}
4.9	No mycorrhiza	0.96^{d}	23^{c}	8^{b}	46^{c}	11^{cd}	0.8^{b}	0.35^{bc}	0.09^{b}	3.9^{b}	0.35^{cd}
	Gi. margarita	1.41^{c}	25^{c}	8^{b}	36^{c}	11^{cd}	1.3^{b}	0.26^{c}	0.07^{b}	3.6^{b}	0.48^{c}
	G. etunicatum	0.75^{d}	22^{c}	8^{b}	37^{c}	10^{cd}	0.7^{b}	0.28^{c}	0.08^{b}	3.9^{b}	0.25^{d}
5.2	No mycorrhiza	1.00^{d}	27^{c}	8^{b}	43^{c}	9^{cd}	1.2^{b}	0.28^{c}	0.11^{b}	3.5^{b}	0.32^{cd}
	Gi. margarita	1.53^{c}	32^{c}	10^{b}	42^{c}	10^{cd}	1.0^{b}	0.25^{c}	0.09^{b}	3.2^{b}	0.25^{d}
	G. etunicatum	0.81^{d}	28^{c}	8^{b}	40^{c}	8^{d}	0.9^{b}	0.27^{c}	0.08^{b}	3.4^{b}	0.25^{d}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$

3.4 Discussion

The results show that low pH affected root colonization and contributions of AM fungi to the growth of cowpea. As Al concentrations existed at sub-toxic levels and other elements were relatively stable, the results clearly indicate that the excessive concentration of H^+ ions was the major factor for inhibition of mycorrhizal fungi in the growth medium. This supports the report of Porter *et al.* (1987b) regarding the potential effect of high H^+ ion concentration in inhibiting the growth and function of AM fungi under acidic soil conditions.

In general, these results clearly demonstrate the important roles of AM fungi in assisting plants to grow better in acidic soils as has previously been reported (Raju *et al.,* 1988; Clark *et al.,* 1999; Nurleany *et al.,* 1996). Depressed growth of non-mycorrhizal cowpea at pH 4.6 was reversed to a greater extent by inoculating with AM fungi than by increasing the pH, so that the growth of plants with mycorrhizas at pH 4.6 exceeded that without mycorrhiza at pH 5.2 (Table 3.2).

Element concentrations in plant tissues were affected differently by mycorrhizal inoculation. Some increased (e.g. Ca, Mg and S in shoots and P and Na in roots) or decreased (e.g. Fe, Na and Al in shoots and K and Zn in roots), but most did not change due to the symbiosis (Tables 3.3 and 3.4). In general, these elements were in sufficient concentrations particularly in shoots for adequate nutrition, except P that was slightly deficient (Reuter and Robinson, 1997). P deficiency particularly in shoots of mycorrhizal plants possibly resulted from utilization of acquired P for growth, so that no luxury

accumulation to high concentrations in the tissues occurred. On the other hand, mycorrhiza formation increased total nutrient uptake by the plants (Table 3.5) and in some cases also increased element uptake efficiency (Table 3.7). Mycorrhizal plants had greater uptake of most elements measured, particularly at pH 4.6, where the growth medium was less conducive to plant growth. These findings are in line with the results of Raju *et al.* (1988) in sorghum and Clark (2002) in switchgrass (*Panicum virgatum* L.), who reported that AM fungi increased element uptake per unit root length in acid soils. Sanders and Tinker (1973) firstly used the amount of element uptake per unit root length to compare uptake efficiency of nutrients between mycorrhizal and non-mycorrhizal plants. Therefore, the improvement of cowpea growth by mycorrhizas at low pH could be a consequence of increased nutrient acquisition (Muthukumar and Udaiyan, 2000). In contrast, as increasing pH of growth media improved plant growth, the contributions of mycorrhizal fungi to mycorrhizal growth response and element uptake efficiency decreased dramatically (Tables 3.2 and 3.6).

This study also found that there was a big difference between the two AM fungi tested in responding to low pH. *Gi. margarita* was well adapted to low pH, and accordingly had high root colonization and a great effect on enhancing plant growth (Fig. 3.1 and Table 3.2) and nutrient uptake (Table 3.5). This confirms results of previous work by Siqueira *et al.* (1984) who found that low pH did not affect spore germination and germ tube growth of *Gi. margarita,* and by Yawney *et al.* (1982), who found that the fungus significantly improved the growth and element uptake of sweet gum

(*Liquidambar styraciflua* L.) seedlings grown at pH 4.5, with a smaller improvement being observed at higher pH. In contrast, the ability of *G. etunicatum* to colonize roots decreased with decreasing soil pH (Fig. 3.1) and so had no significant effect on cowpea plant growth (Table 3.2). The ineffectiveness of *G. etunicatum* is inconsistent with some recent findings demonstrating considerable effects of the fungus on the growth and nutrient uptake of other plant species grown in acidic soil conditions (Clark *et al.*, 1999; Clark, 2002), even when soluble Al ions were present at a high level (Borie & Rubio 1999). The inconsistency could result from variations in the physiology of the fungal isolates, plant species or experimental procedure and conditions used (Jakobsen *et al.*, 1992a, b). The difference in pH responses by *Gi. margarita* and *G. etunicatum* have also been demonstrated by Clark *et al.* (1999) when inoculating switchgrass in soils with pH_{Ca} 4 and 5. Root colonization by *Gi. margarita* was higher than *G. etunicatum* at pH 4, but was not different at pH 5.

Compared to *G. etunicatum*, the growth improvement by *Gi. margarita* might be attributed to increased root growth in terms of root length (Table 3.2). Good development of the root system is essential for plants to tolerate acid soils (Miyasaka and Habte, 2001) because this increases the plant access to nutrients in these soils. In addition, the higher plant growth response to *Gi. margarita* could also result from the activities of external hyphae, since the fungus has good tolerance to low pH (Bartolome-Esteban and Schenck, 1994; *see* also Chapter 5). Because external hyphae were not

measured in this experiment it is difficult to make conclusions regarding the function of the hyphae; therefore further work is required.

In conclusion, low pH markedly interfered with the effects of AM fungi on plant growth. The extent of the mycorrhizal effects at low pH was strongly dependent on fungal species. *Gi. margarita* was much more tolerant of low pH and so more infective and effective than *G. etunicatum* as this fungus had no effect on the plant growth.

The ability of *Gi. margarita* to colonize roots and to increase nutrient uptake and plant growth at low pH means that it may also have potential to ameliorate the effects of excessive aluminium. This is not likely to be the case for *G. etunicatum*.

CHAPTER 4

MYCORRHIZAL PLANT RESPONSES TO ALUMINIUM

4.1 Introduction

In a previous experiment (Chapter 3) *Gi. margarita* showed the ability to accommodate stress conditions of low pH and make significant contributions to growth and nutrient uptake of cowpea, indicating that the fungus has good tolerance of excess H^+ ion activity. Nevertheless, it is not known whether the fungus remains effective when high soluble Al exists in the growth medium.

This chapter presents the results of an experiment carried out to assess the effects of Al on the growth of *Gi. margarita* and its role in cowpea growth and element uptake. Included in this experiment was an Al concentration of about 4 ppm that reduced growth of cowpea by about 30% compared to that of control plants (Fig. 2.1).

4.2 Materials and methods

This experiment was conducted under glasshouse conditions during December 2000 to February 2001. It was a factorial experiment with a completely randomised design. There were four levels of soluble Al concentrations (1.1, 4.1, 7.3 and 11.9 ppm in growth media A_1, A_4, A_7 and A_{12} resulting from addition of 0, 75, 150 and 300 μg Al g^{-1} medium

respectively at pH 4.7, *see* Section 2.1 for detail), and two levels of inoculation with (M) and without (NM) mycorrhizal spores. There were 5 replicates and 1 extra pot for each treatment.

The cowpea cultivar, *Gi. margarita* isolate and growth media used in this experiment are described in Chapter 2.

Pre-germinated cowpea seeds were transplanted singly into pots containing 700 g of growth medium with or without inoculation with 250 spores of the fungus Pots without plants and inoculation were included as control treatments. Soil moisture and pH in growth media were maintained by watering to weight as needed during the experiment with RO water adjusted with H_2SO_4 to pH 4.7. Plants were grown for 6 weeks before harvest.

At harvest, shoot and root dry weights, mycorrhizal colonization, element uptake and media pH were measured using procedures described in Chapter 2. Plants in extra pots had been harvested 2 weeks after planting to observe Al toxicity symptoms in plant roots.

Data were analyzed using ANOVA to determine significant differences between the effect of treatments, and tested based on LSD values at $P \leq 0.05$ (*see* Section 2.12). No data could be collected from treatment A_{12} as most plants in this growth medium died; consequently, only data from A_1, A_4 and A_7 with and without mycorrhiza were analyzed statistically and presented. To determine mycorrhizal plant response, a comparison was made between mycorrhizal and non-mycorrhizal plants at every Al concentration.

4.3 Results

4.3.1 Growth media pH

The pH of growth media in planted pots both with and without mycorrhizal inoculation was measured after the experiment and was not significantly different from those in unplanted control pots; they all increased slightly during the experiment (Table 4.1).

Table 4.1 Growth media pH measured before and after the experiment

		Growth medium			
		A_1	A_4	A_7	A_{12}
Before experiment		4.73	4.69	4.74	4.70
After experiment					
Unplanted pots		4.81	4.80	4.79	4.82
Planted pots:	NM	4.84	4.78	4.80	4.81
	M	4.79	4.82	4.79	4.78

A_1, A_4, A_7 and A_{12} are growth media containing 1.1, 4.1, 7.3 and 11.9 ppm soluble Al; NM (non-mycorrhiza) and M (mycorrhiza).

4.3.2 Plant growth

The presence of soluble Al in growth media considerably affected plant growth. The toxic effect of Al increased with concentration (Figs. 4.1 and 4.2). At a concentration of about 12 ppm soluble Al in soil, plants died within two weeks after transplanting regardless of mycorrhizal inoculation.

Compared to plant performance in the growth medium with the lowest Al concentration (1.1 ppm, A_1), the dead plants showed symptoms such as yellowing or necrotic spots widening on the leaf lamina and small root systems with a lack of lateral roots, indicating that they were suffering from a complex nutrient disorder.

(a) (b) (c)

Figure 4.1 Shoots and roots of cowpea plants 14 days after planting.
(a) and (b) plants grown in A_1 and A_{12}; (c) roots of plants grown in A_1, A_4, A_7 and A_{12}

Mycorrhizal inoculation increased plant growth, although the effectiveness varied with Al concentration. In general, mycorrhizal plants grew better than non-mycorrhizal plants. Shoot and root dry weights, root length and shoot/root dry weight ratios of mycorrhizal plants were higher than non-mycorrhizal plants. However, the effects were only significant in A_1, the growth medium that contained 1.1 ppm soluble Al. Plant growth responses to mycorrhiza were reduced when soluble Al concentration increased (Table 4.2).

Table 4.2 Growth of cowpea plants with and without mycorrhiza in growth media differing in soluble Al concentrations

Growth medium	Mycorrhizal inoculation	Dry weights of			Shoot/ Root ratio	Root length (m)	MGR (%)
		Shoots	Roots	Plant			
		(g)					
A_1	NM	0.19^{c}	0.11^{a}	0.30^{b}	1.8^{c}	2.9^{b}	0
	M	0.40^{a}	0.16^{a}	0.56^{a}	2.6^{a}	3.8^{a}	85
A_4	NM	0.23^{bc}	0.11^{a}	0.34^{b}	2.1^{bc}	1.8^{c}	0
	M	0.29^{b}	0.12^{a}	0.42^{ab}	2.4^{ab}	2.4^{b}	25
A_7	NM	0.07^{d}	0.03^{b}	0.10^{c}	2.3^{ab}	0.7^{d}	0
	M	0.09^{d}	0.04^{b}	0.13^{c}	2.2^{abc}	0.9^{d}	21

Figures in the same column followed by the same superscripts are not significantly different based on LSD tests at $P \leq 0.05$; A_1, A_4 and A_7 are growth media containing 1.1, 4.1 and 7.3 ppm soluble Al; NM (non-mycorrhiza) and M (mycorrhiza).
Mycorrhizal growth response (%MGR) = [{Plant $DW_{(M)}$ – Plant $DW_{(NM)}$} / Plant $DW_{(NM)}$] x 100

4.3.3 Mycorrhizal colonization

Root colonization by *Gi. margarita* was significantly affected by Al concentration (Fig. 4.2). The percentage of root length colonized by the fungus decreased as Al concentration was increased.

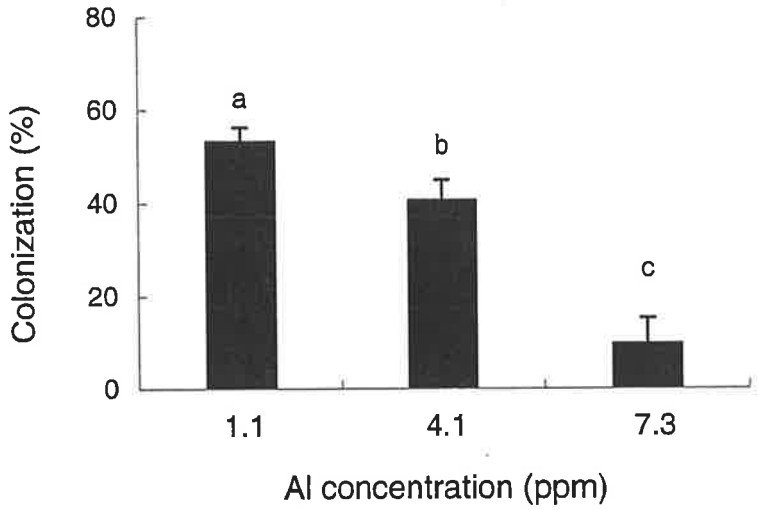

Figure 4.2 Root colonization by *G. margarita* at different Al concentrations

Bars marked with the same letter are not significantly different at P≤0.05;
Bars represent means and standard errors of means (n=5).

4.3.4 Element concentrations in plant tissues

Tables 4.3 and 4.4 present element concentrations in shoots and roots of cowpea plants inoculated with and without spores of *Gi. margarita* in growth media containing different Al concentrations

In general, the fungal inoculation had no significant effects on concentration of elements either shoots or roots irrespective Al concentration, except increased root concentration of P in A_1 and A_4, and of Al in A_1, and decreased shoot concentrations of Zn and Mn in A_4.

Table 4.3 Element concentration in shoot tissues of mycorrhizal and non-mycorrhizal cowpea grown in growth media differing in soluble Al concentrations

Growth medium	Mycorrhizal inoculation	Element concentration									
		P (%)	Ca (%)	Mg (%)	K (%)	S (%)	Fe (mg/g)	Zn (mg/g)	Mn (mg/g)	Na (%)	Al (mg/g)
A_1	NM	0.10^{a}	2.6^{ab}	1.2^{a}	9^{ab}	0.7^{b}	0.50^{ab}	0.25^{a}	0.69^{a}	0.7^{c}	0.25^{ab}
	M	0.12^{a}	3.3^{a}	1.2^{a}	10^{a}	0.9^{b}	0.36^{b}	0.20^{ab}	0.51^{ab}	0.1^{c}	0.19^{b}
A_4	NM	0.08^{a}	2.2^{b}	1.1^{a}	11^{a}	1.0^{b}	0.35^{b}	0.16^{b}	0.63^{a}	2.7^{b}	0.25^{ab}
	M	0.10^{a}	2.1^{b}	0.9^{ab}	9^{ab}	1.1^{b}	0.27^{b}	0.08^{c}	0.37^{bc}	1.5^{bc}	0.22^{b}
A_7	NM	0.10^{a}	1.2^{c}	0.7^{b}	7^{ab}	2.8^{a}	0.82^{a}	0.07^{c}	0.32^{c}	8.7^{a}	0.33^{ab}
	M	0.10^{a}	1.0^{c}	0.5^{b}	5^{b}	2.3^{a}	0.57^{ab}	0.05^{c}	0.28^{c}	7.6^{a}	0.52^{a}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$;
A_1, A_4 and A_7 are growth media containing 1.1, 4.1 and 7.3 ppm soluble Al;
NM (non-mycorrhiza) and M (mycorrhiza).

Table 4.4 Element concentration in root tissues of mycorrhizal and non-mycorrhizal cowpea grown in growth media differing in soluble Al concentrations

Growth medium	Mycorrhizal inoculation	Element concentration									
		P (%)	Ca (%)	Mg (%)	K (%)	S (%)	Fe (mg/g)	Zn (mg/g)	Mn (mg/g)	Na (%)	Al (mg/g)
A_1	NM	0.11^{c}	0.7^{a}	0.42^{c}	15^{a}	2.1^{c}	1.4^{ab}	0.44^{a}	0.06^{a}	0.7^{d}	5^{c}
	M	0.20^{ab}	1.1^{a}	0.73^{a}	14^{ab}	3.0^{bc}	1.4^{ab}	0.47^{a}	0.06^{a}	1.7^{cd}	11^{b}
A_4	NM	0.11^{c}	0.9^{a}	0.61^{bc}	14^{ab}	3.4^{ab}	0.6^{b}	0.22^{bc}	0.05^{a}	3.2^{bc}	12^{b}
	M	0.27^{a}	1.0^{a}	0.63^{bc}	10^{b}	3.0^{bc}	0.7^{b}	0.32^{ab}	0.05^{a}	3.8^{b}	15^{ab}
A_7	NM	0.17^{bc}	0.7^{a}	0.71^{a}	5^{c}	4.2^{a}	2.1^{a}	0.15^{c}	0.05^{a}	6.1^{a}	19^{a}
	M	0.18^{bc}	1.0^{a}	0.85^{a}	5^{c}	3.7^{ab}	1.3^{ab}	0.20^{bc}	0.05^{a}	4.9^{ab}	5^{ab}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$;
A_1, A_4 and A_7 are growth media containing 1.1, 4.1 and 7.3 ppm soluble Al;
NM (non-mycorrhiza) and M (mycorrhiza).

4.3.5 Element uptake

The ability of plants to take up elements was greatly influenced by Al concentration. Increases in Al also significantly affected mycorrhizal contribution to the uptake efficiency. However, as shown in Table 4.5, there was considerable variation in the effects of Al and of mycorrhizal inoculation.

For non-mycorrhizal plants, the presence of soluble Al at 4.1 ppm (A_4) had no significant effects on element uptake except for Na and Al, where uptake increased compared with that in A_1. Increasing the Al concentration in the growth medium to 7.3 ppm (A_7) significantly reduced P, Ca, Mg, K, Zn and Mn uptake, but did not affect S, Fe, Na and Al uptake. For mycorrhizal plants, the uptake of P, Ca, Mg, K, Zn and Mn was significantly lower in plants grown in A_4 and A_7 than in A_1. The uptake of S and Al was not different in A_1 and A_4, but significantly lower in A_7. The uptake of Fe decreased greatly in A_4 but no further significant decrease was observed in A_7, whilst the uptake of Na increased markedly in A_4 and then remained unchanged in A_7.

Table 4.5 also shows that in general, compared to those plants without mycorrhiza, element uptake by plants with mycorrhiza was significantly higher for P, Ca, Mg, K, S, Zn, Mn and Al in A_1, but only for P in A_4; no other significant differences between mycorrhizal and non-mycorrhizal plants were found

The element uptake response of plants to mycorrhiza is summarized in Table 4.6. It seems that *Gi. margarita* significantly increased element uptake only in the growth medium with low soluble Al (A_1). In this medium,

the highest increase was Al (245%) followed by Ca (161%) and P (156%). Additional increases in Al concentrations reduced the contributions of the fungus. Negative responses to mycorrhiza were also observed for some elements including K, Fe, Mn, Na and Al, although these were not significant when data on their total uptake by mycorrhizal and non-mycorrhizal plants at equivalent Al level were compared (*see* Table 4.5).

Table 4.5 Element uptake by mycorrhizal and non-mycorrhizal cowpea plants grown in growth media differing in soluble Al concentrations

Growth medium	Mycorrhizal inoculation	Element uptake (mg plant^{-1})									
		P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
A_1	NM	0.32^{c}	5.8^{b}	2.7^{b}	32.7^{b}	3.7^{c}	0.26^{ab}	0.09^{b}	0.14^{b}	2.1^{b}	0.5^{c}
	M	0.82^{a}	15.2^{a}	6.0^{a}	62.8^{a}	8.7^{a}	0.35^{a}	0.16^{a}	0.22^{a}	3.1^{b}	1.8^{a}
A_4	NM	0.35^{c}	5.4^{b}	3.0^{b}	38.5^{b}	5.8^{bc}	0.14^{ab}	0.06^{b}	0.14^{b}	9.2^{a}	1.4^{ab}
	M	0.68^{b}	7.2^{b}	3.4^{b}	38.6^{b}	6.9^{ab}	0.16^{b}	0.06^{b}	0.12^{b}	8.7^{a}	1.9^{a}
A_7	NM	0.14^{d}	1.1^{c}	0.7^{c}	6.8^{c}	3.4^{c}	0.13^{ab}	0.01^{c}	0.02^{c}	8.2^{a}	0.6^{bc}
	M	0.16^{d}	1.3^{c}	0.8^{c}	6.7^{c}	3.4^{c}	0.10^{b}	0.01^{c}	0.03^{c}	8.4^{a}	0.6^{bc}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$;
A_1, A_4 and A_7 are growth media containing 1.1, 4.1 and 7.3 ppm soluble Al;
NM (non-mycorrhiza) and M (mycorrhiza).

Table 4.6 Mycorrhizal element uptake responses of cowpea plants grown in growth media differing in soluble Al concentrations

Growth medium	Element uptake (% response)									
	P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
A_1	156*	161*	119*	92*	136*	35*	66*	56*	51	245*
A_4	94*	32	12	0	19	11	6	(-15)	(-5)	43
A_7	13	12	8	(-1)	0	(-18)	22	4	3	(-3)

A_1, A_4 and A_7 are growth media containing 1.1, 4.1 and 7.3 ppm soluble Al.
Mycorrhizal element uptake response (%) = [{$Uptake_{(M)}$-$Uptake_{(NM)}$} / $Uptake_{(NM)}$] x 100
Values marked with * are based on values significantly different each other in Table 4.6.

The effect of mycorrhizal inoculation on the uptake efficiency of plant roots (specific root uptake) varied extensively with elements, and was affected by Al concentration as shown in Table 4.7. It appeared that the function of mycorrhiza in increasing uptake efficiency of host plant roots was generally evident only in A_1. Although mycorrhizal plants were generally more efficient than non-mycorrhizal plants, significant increases were observed only for P, Ca and Mg uptake. On the other hand, at higher Al concentrations (A_4 and A_7) uptake efficiency was generally the same in mycorrhizal and non-mycorrhizal plants, except for P and Mn in A_4, and Na and Al in A_7.

Table 4.7 Element uptake per unit root length of mycorrhizal and non-mycorrhizal cowpea plants grown in growth media differing in soluble Al concentrations

Growth medium	Mycorrhizal inoculation	Element uptake ($\mu g\ cm^{-1}$ root length)									
		P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
A_1	NM	1.1^{c}	21^{bc}	9^{b}	113^{bc}	13^{d}	0.9^{b}	0.32^{ab}	0.48^{bc}	7^{d}	1.9^{c}
	M	2.2^{ab}	40^{a}	16^{a}	165^{ab}	23^{cd}	0.9^{b}	0.41^{a}	0.58^{ab}	8^{d}	4.9^{c}
A_4	NM	1.9^{bc}	30^{ab}	17^{a}	214^{a}	32^{bc}	0.8^{b}	0.32^{ab}	0.78^{a}	51^{c}	7.6^{ab}
	M	2.8^{a}	30^{ab}	14^{ab}	161^{ab}	29^{bc}	0.7^{b}	0.25^{bc}	0.50^{bc}	36^{c}	8.1^{ab}
A_7	NM	2.0^{ab}	16^{c}	10^{b}	97^{c}	49^{a}	1.9^{a}	0.14^{c}	0.29^{c}	117^{a}	9.1^{a}
	M	1.8^{bc}	14^{c}	9^{b}	74^{c}	38^{ab}	1.1^{ab}	0.13^{c}	0.33^{c}	93^{b}	6.9^{b}

Figures in the same column followed by the same superscripts are not significantly different at $P \leq 0.05$;
A_1, A_4 and A_7 are growth media containing 1.1, 4.1 and 7.3 ppm soluble Al;
NM (non-mycorrhiza) and M (mycorrhiza).

4.4 Discussion

This experiment demonstrates that excess Al in growth media severely affected both the growth of cowpea plants and the development and function of mycorrhiza formed by *Gi. margarita*. As the pH of growth media was successfully controlled by watering with RO water at pH 4.7 the effects can be attributed to Al rather than the effects of pH.

Particularly for non-mycorrhizal plants, increased concentrations of soluble Al in growth media reduced root dry weight and root length. Root development was very poor when soluble Al exceeded 4 ppm irrespective of mycorrhizal colonization (Fig. 4.1; Table 4.2) and this in turn seems to result in poor growth of plants in general (Table 4.2). This experiment suggested that at a concentration of about 4 ppm, soluble Al in the growth medium affected the concentrations of some elements in shoots and roots differently (Tables 4.3 and 4.4), but did not affect total uptake of most of the elements measured, although increasing soil Al to 7.3 ppm markedly reduced uptake of most elements (Table 4.5). The increases in uptake of Al (in A_4) and of Na (in A_4 and A_7) may simply reflect their increased concentrations in the growth medium (*see* Table 2.2). Toxicity due to the high tissue Na concentrations resulting from the high Na in treatments A4 and A7 cannot be ruled out. The issue of separating toxic effects of high concentration of Na in plants as a cause of the decreased growth, rather than the high Al *per se*, will require further investigation. For the rest of this thesis the effects are attributed to high Al and mostly discussed accordingly.

The inhibitory effect of Al on cowpea plants confirms the result of a preliminary experiment (Fig. 2.1), demonstrating that Al above concentrations of about 4 ppm soluble Al at pH 4.6 is toxic to the plants. About 12 ppm soluble Al killed all young plants within two weeks after transplanting. These results indicate that the cowpea cultivar 'Red Caloona' has a limited tolerance to Al toxicity, particularly when seeds were directly sown in growth media treated with high Al. It is likely that excess Al detrimentally affected primary roots growing from germinating seeds, resulting in a poorly developed root system (Fig. 4.1c) and producing poor growth. The damaging effect of Al on roots of seedlings of other plants species has been shown by previous studies, mainly using solution cultures. Inhibition of root elongation induced by Al can occur very rapidly, even in hours (Care, 1995; Llugany *et al.*, 1995; Massot *et al.*, 1999). Therefore, in relation to Al toxicity, the first few days seems to be a critical period for the young plants, and this can be an important aspect to be considered for agronomic practices. For further experiments, the technique of transplanting should be modified by not growing the plant seeds directly in Al-treated media but firstly in a growth medium of low Al for 7-14 days prior to transplanting. This will increase resistance of young plants to Al toxicity (Baligar *et al.*, 1995).

For the fungus, increasing Al concentrations markedly reduced the percentages of root length colonized from 53% in A_1 to 40% in A_4 and to only 10% in A_7, This suggests that although *Gi. margarita* is capable of functioning at low pH (Bartolome-Esteban and Schenck, 1994; *see* also results in

Chapter 3), the presence of high soluble Al under acidic conditions restricted its ability to colonize roots.

It is important to note that in relation to Al effects, mycorrhizal inoculation with *Gi. margarita* spores only made significant contributions to the growth of plants in the growth medium containing about 1 ppm soluble Al, in which the plant growth response to mycorrhiza was 85%. The response decreased to 25 and 21% (Table 4.2) with increasing Al concentrations to about 4 and 7 ppm. This suggests that at Al concentrations typically toxic to cowpea (4 ppm or higher; Fig. 2.1 in Chapter 2) *Gi. margarita* was not effective in alleviation of Al toxicity to the plant. Compared to that at pH 4.6 in the experiment in Chapter 3 (*see* Table 3.2) the contribution of the fungus to plant growth in A_1 was much lower. This difference might be related to growth of non-mycorrhizal control plants that was doubled in this experiment compared to that in previous experiment, whilst the growth of mycorrhizal plants in the two experiments was not different (*see* Tables 3.2 and 4.2). Furthermore, the low growth response of cowpea to mycorrhiza particularly at A_4 and A_7 might be related to the sensitivity of cowpea plant itself to Al stress. Depression in growth caused by Al may have resulted in plants unable to support mycorrhizal function as already shown by Borie and Rubio (1999) for two barley cultivars differing in Al responses.

Following the plant growth response, significant element uptake responses to the symbiosis also occurred in A_1 for most of the elements, but only for P in A_4 (Tables 4.5 and 4.6). This shows the consistency of mycorrhizal function in improving P uptake by plants including acidic soil

conditions (Raju *et al.*, 1988; Medeiros *et al.*, 1994; Clark and Zeto, 1996a, 2000) in which the element is often unavailable to plants due to fixation by Al in soil (Marschner, 1991; Baligar *et al.*, 1995). In addition to P, improvement of mycorrhizal cowpea growth might also be attributed to increased uptake of Ca (Tables 4.5 and 4.6). This element is essential for root elongation in response to Al toxicity (Sanzonowicz *et al.*, 1998; Pintro *et al.*, 1998), and has been shown involved in mechanisms for alleviation of Al toxicity (*see* Foy, 1992; Alva *et al.*, 1986a; Reid *et al.*, 1995; Kinraide, 1998).

Interestingly, in this experiment the highest increase in element uptake by mycorrhiza of about 250% was observed for Al in A_1 (Table 4.6). It seems that the increase was related to increased plant growth (Table 4.2) and higher Al concentration in plant roots (Table 4.4). Previous work has shown that mycorrhizal inoculation increased (Yawney *et al.*, 1982; Medeiros *et al.*, 1994; Clark, 2002), decreased (Koslowsky and Boerner, 1989; Clark and Zeto, 1996a) or did not change (Rufyikiri *et al.*, 2000) Al uptake by plants.

Although percentage colonization does not always correlate closely with mycorrhizal role in plant growth (Smith and Read, 1997; Clark *et al.*, 1999; Smith *et al.*, 2000) this experiment showed that under Al stress, mycorrhizal growth response was only significant when percentage root colonization was about 55%, and decreased as root colonization decreased. That mycorrhizal colonization was unable to compensate for poor root growth under conditions of this experiment contrasts with the observations of Clark *et al.* (1999) and Clark (2002), who reported that *Gi. margarita* is very effective in increasing the growth and element uptake of switchgrass

(*Panicum virgatum*) grown in an acidic soil (pH_{H2O} 4.5) containing 302 ppm 1M KCl extractable-Al and 88% Al saturation. Although not strictly comparable due to many differences in experimental conditions, differences in the fungal performance could be attributed to differences in the interactions between the host plant and the fungus, particularly in relation to the responsiveness of plants to mycorrhizal colonization (Yost and Fox, 1979). It has been considered that switchgrass is highly responsive (Hetrick *et al.*, 1987) while cowpea is only moderately dependent on mycorrhiza (Yost and Fox, 1979; Muthukumar and Udaiyan, 2000; Tables 4.2 and 4.6).

In general, the effect of Al on the growth and function of AM fungi remains unresolved. High Al concentrations in growth media may negatively affect root colonization and mycorrhizal contributions. On the other hand, several AM fungal species show some ability to mitigate Al toxicity and improve plant growth and nutrient uptake (*see* Chapter 1). So far, it remains largely unknown how AM fungi interact with Al toxicity since only few studies have been made in this area and the variation in experimental materials and procedures makes the results difficult to compare (*see* a review by Habte, 1999; Clark, 1997). However, it has been shown that the physiology of host plants and the tolerance of AM fungi to Al make significant contributions to the outcomes of the interactions (Mendoza and Borie, 1998: Borie and Rubio, 1999; Koslowsky and Boerner, 1989).

Reduction in root colonization and so mycorrhizal effectiveness observed in this experiment could possibly be the results of (1) inhibition of spore germination and/or germ tube growth of the fungus, since these early

stages in fungal life are very sensitive to environmental stresses including Al toxicity; and/or (2) a poorly developed root system induced by Al toxicity (Figs. 4.1c and Table 4.2). Small root systems provide fewer infection sites for fungal colonization and this would also limit the root base of the fungus for carbon acquisition and further growth. Thus a third confounding factor may be the inhibited external hyphal growth that was possibly caused by either Al toxicity or by reduced support in terms of sugar supply from host plants already suffering from Al toxicity. Limited hyphal network development on one hand reduces the potential of an inoculum to colonize new roots, and on the other hand reduces the capacity of mycorrhizal root systems to explore and take up elements beyond root depletion zones in particular. However, these possibilities need to be investigated further.

In general, it can be concluded that the presence of increasing concentrations of soluble Al in growth medium reduced the percentage of root length colonized by *Gi. margarita*, this in turn reduced mycorrhizal function in the growth of cowpea plants. At concentrations of Al toxic to cowpea (4 ppm or above) the fungus had only a small effect in increasing plant growth and element uptake. There is a need, therefore, to investigate whether reduced root colonization by Al relates to inhibited early growth of the fungus including spore germination and germ tube growth, or to inhibited growth and spread of external hyphae that in turn impaired their contribution to host plant growth.

CHAPTER 5

EFFECTS OF ALUMINIUM ON THE EARLY GROWTH OF *Gigaspora margarita*

5.1 Introduction

Species or isolates of AM fungi have been shown to prefer certain soil pH ranges. Many species are inhibited at low pH but others are favoured by low pH (Clark and Zeto, 1996b; Clark, 1997). However, in most cases the pH preferences have not been addressed clearly at particular stages in the life cycle of the fungus (*see* Fig. 1.2). It is believed that every stage of the life cycle may have a different response or sensitivity to factors associated with soil acidity (*see* Robson and Abbott, 1989; Habte, 1999).

Spore germination and hyphal growth are critical stages in mycorrhiza formation and determine whether an AM fungus will succeed or fail to initiate and establish the symbiosis (Giovannetti, 2000). Although these stages are typically dependent on the intrinsic condition of the spores, a number of stress factors in soil can inhibit them to some extent (*see* Section 1.2.2). Some early work showed that both spore germination and germ tube elongation of some species of AM fungi were inhibited at low pH *per se* or by excessive Al concentrations in growth media (Green *et al.*, 1976; Bartolome-Esteban and Schenck, 1994). Therefore, low mycorrhizal colonization in acidic soils was often attributed to reduction in the early stages of fungal growth (Robson and Abbott, 1989). However, the relation between the extent

of root colonization and inhibition of the early growth of the fungus has not been demonstrated directly.

Previous chapters show that root colonization by *Gi. margarita* was not affected by a low pH of 4.6 (Chapter 3), but decreased when soluble Al existed at concentrations of about 4 ppm or above (Chapter 4). Thus, it is interesting to investigate further if Al has direct effects on the early growth of the fungus.

Four experiments were carried out. Aims, Material and Methods, and Results are presented separately. The Discussion covers all experiments together.

5.2 Experiment 1 Effects of low pH on spore germination and germ tube growth

5.2.1 Experimental aim

This experiment aimed to study the effects of low pH *in vitro* on spore germination and germ tube elongation of *Gi. margarita*.

5.2.2 Materials and methods

This experiment was carried out in the laboratory, and consisted of three levels of media pH with 5 replications. Spores extracted from pot culture were soaked into a small tube containing antibiotic solution (Penicillin, 100 mg L^{-1} and Streptomycin sulphate, 200 mg L^{-1}) overnight and rinsed three times in sterile deionized water prior to placing on plates in Petri dishes. The plates

were prepared using 20 mL of 0.8% Phytagel in deionized water and adjusted to pH 4.5, 4.8 and 5.3 using 0.1 M HCl. Ten spores were aseptically transferred on to a plate, five plates per pH treatment. Spore germination and hyphal growth were observed after a 10-day incubation period at 23°C in the dark. A spore was considered as germinated when generated one or more germ tubes. Hyphal length produced by germinated spores was assessed based on a gridline intersect method (Giovannetti and Mosse, 1980) using an eyepiece graticule as for external hyphae (Section 2.9).

Data were analyzed using ANOVA followed by LSD tests at $P \leq 0.05$. Data on spore germination and hyphal length are presented in percent and in mm per spore respectively.

5.2.3 Results

Spores of *Gi. margarita* germinated within 4-5 days after plating. The spores generated one or more germ tubes through the spore wall. At ten days, most of the germ tubes had become quite extensive and their hyphae elongated up to 35 mm. Auxiliary cells were formed by the hyphae (Fig. 5.1).

Figure 5.2 shows that low pH significantly affected spore germination and hyphal length per spore. No significant difference in spore germination was observed between pH 5.3 and 4.8, but germination was significantly lower at pH 4.5. The growth of hyphae developing from germ tubes decreased significantly with decreasing pH. At pH 4.5 hyphal length was about 14 mm compared to about 37 mm at pH 5.3.

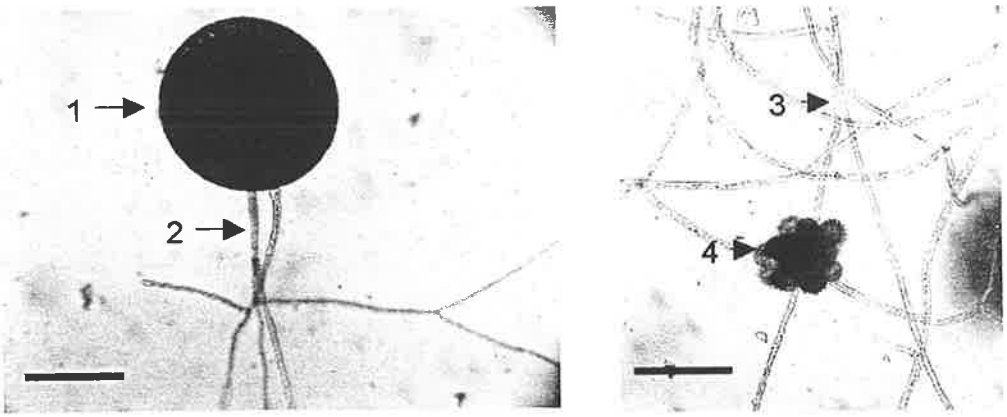

Figure 5.1 (1) Germinated spore, (2) germ tubes, (3) germination hyphae and (4) auxiliary cells of *Gi. margarita* observed at pH 5.3
(Scale bars: 250 μm)

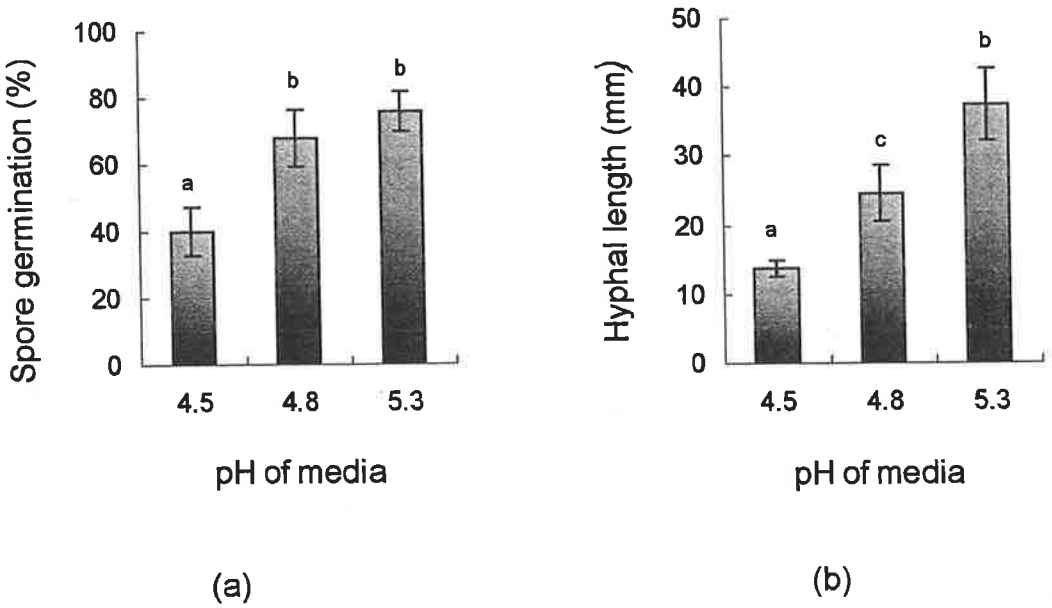

Figure 5.2 (a) Spore germination and (b) hyphal length per spore of *Gi. margarita* at different media pH values

Bars in the same graph marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=5)

5.3 Experiment 2 Effects of Al on spore germination and germ tube growth

5.3.1 Experimental aim

The aim was to assess the effects of Al *in vitro* on spore germination and hyphal growth of *Gi. margarita*.

5.3.2 Materials and methods

The experiment was a 5 x 2 factorial experiment with 5 levels of Al concentrations and 2 levels of media pH. This was replicated 5 times.

The materials and procedure were the same as used in Experiment 1 above, except for treatments given to the Phytagel plates. $AlCl_3$ solution was soaked into the media to give Al concentrations of 0.7, 1.4, 2.7 and 5.4 ppm, and pH of the media were adjusted to 4.8 and 5.3 using HCl solution. Control plates without Al addition were also prepared.

Data were analyzed using ANOVA and LSD tests at $P \leq 0.05$ to determine differences between Al treatments. Inhibitory effects of Al treatments on spore germination and on hyphal growth were determined using the formula below at every Al level for every pH level.

$$\% \text{ inhibition} = \{(A_O - A_X) / A_O\} \times 100$$

A_O = the mean of spore germination or hyphal length at media without added Al

A_X = spore germination or hyphal length in growth medium with an added Al

5.3.3 Results

There was no interacting effect of Al concentration and pH of media on spore germination. Al significantly inhibited the germination, while no significant effect was observed for media pH.

Regardless of pH, the inhibitory effects of Al increased with concentrations in growth media (Fig. 5.3a). Compared to media without Al addition, spore germination was significantly decreased at 2.7 ppm Al or above (Fig. 5.3b). On the other hand, the effect of Al on hyphal growth was highly dependent on pH, and was more pronounced at pH 4.8 than 5.3 (Fig. 5.4a). In addition, at both pH levels a significant reduction in hyphal length by Al consistently occurred as the amount of Al in growth media increased (Fig. 5.4b).

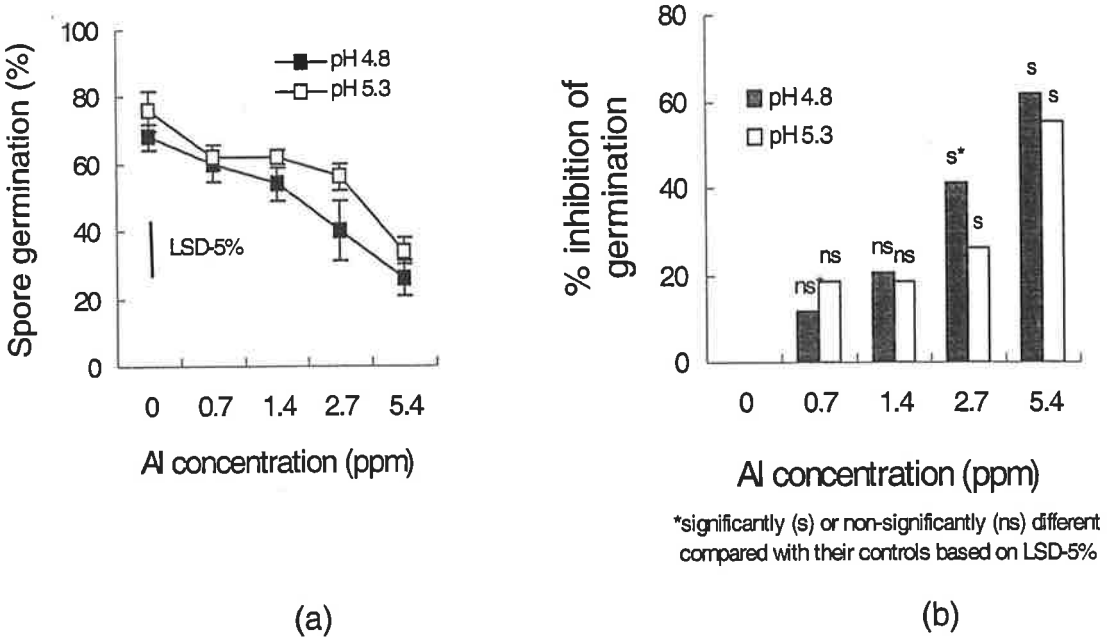

Figure 5.3 (a) Spore germination of *Gi. margarita* and (b) inhibitory effect of Al on the germination at different media pH values

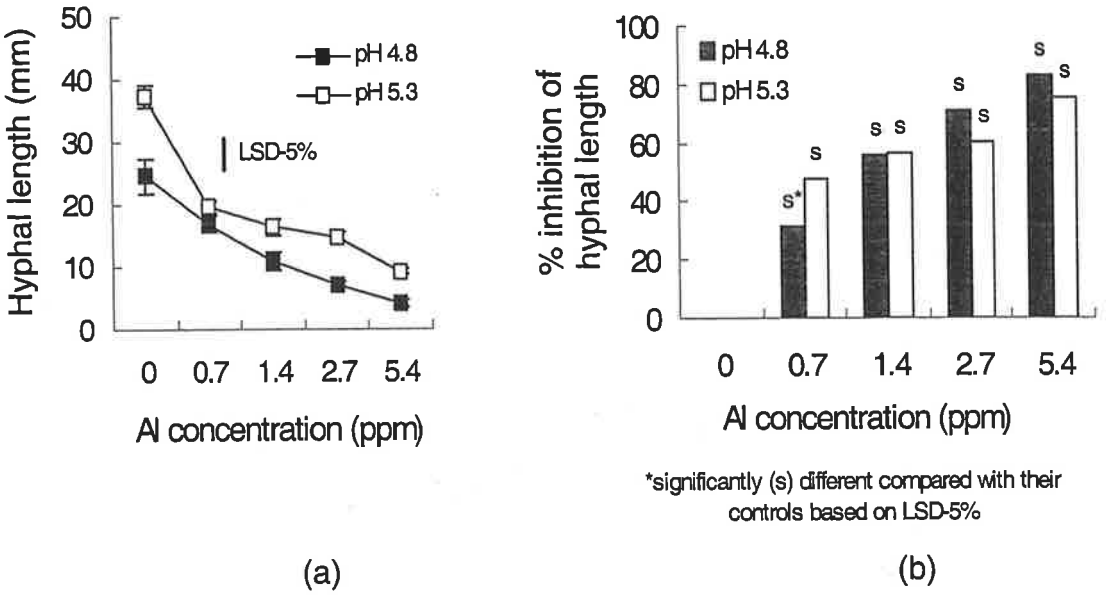

Figure 5.4 (a) Hyphal growth of *Gi. margarita* spores and (b) inhibitory effect of Al on the hyphae at different media pH values

5.4 Experiment 3 Effect of Al on spore germination in soil

5.4.1 Experimental aim

The aim of this experiment was to assess the effect of Al concentrations on the spore germination of *Gi. margarita* in soil.

5.4.2 Materials and methods

This experiment was carried out in 9 cm diameter Petri dishes using sterilized media of sand and soil (90:10, w/w) mixtures differing in soluble Al concentrations. Al concentrations of 1.1, 4.1, 7.3 and 11.9 ppm in media A_1, A_4, A_7 and A_{12} were included with 0.4 ppm in medium M_O (pH 5.3) as a control (*see* details in Section 2.1). The soil depth was 1.0 cm. There were five replicates for every treatment.

Thirty to forty surface-disinfected spores were sandwiched between two 25 mm diameter Millipore® filters, and then buried 0.5 cm deep (midway). Soil pH of 4.7 and moisture content of 0.2 g g^{-1} soil were maintained throughout the incubation period, using sterilized RO water with the pH adjusted to 4.7 with H_2SO_4, whilst the control (M_O) just received sterilized RO water.

Spore germination was observed three weeks after incubation at 25°C in the dark. Inhibitory effects of Al treatments on spore germination were also calculated with the method used in Experiment 2 above.

5.4.3 Results

Germination of *Gi. margarita* spores in soil was significantly affected by Al (Fig. 5.5). The inhibitory effects of Al on germination increased with increasing concentrations in soil. The significant reduction in spore germination mainly occurred when Al concentration increased from 1.1 ppm to 7.3 ppm, and then remained steady at 11.9 ppm.

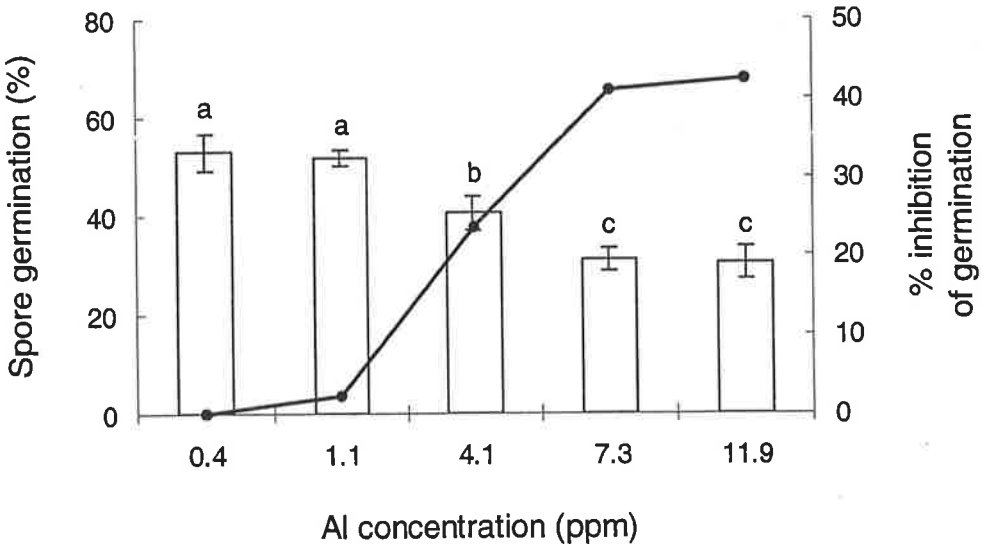

Figure 5.5 Spore germination of *Gi. margarita* (□) and inhibitory effect of Al concentration on the germination (—) in soil media

Bars marked with the same letters are not significantly different at $P \leq 0.05$.
Bars represent means and standard errors of means (n=5).

5.5 Experiment 4 Effect of Al on spore infectivity in soil

5.5.1 Experimental aim

This experiment aimed to assess the effect of Al on spore infectivity of *Gi. margarita* in terms of the ability of the spores to grow and colonize host plant roots. As different plant P status may have different effects on spore growth stimulation and/or root colonization (*see* Tawaraya *et al.*, 1998; Amijee *et al.*, 1989) this experiment also looked at the effect of host plants grown with and without P addition on the spore infectivity. A compartmented pot system that enables these effects to be investigated independently was used.

5.5.2 Materials and Methods

This experiment was conducted under glasshouse conditions in December 2001 - February 2002. The compartmented pot system used is illustrated in Fig. 5.6. Plants, used as trap plants, were grown in cylindrical mesh bags (30μm mesh, 3 cm diameter, 8 cm high) in a Plant Compartment (PC) to prevent root to grow into the outer compartment that was provided for spore germination and germ tube growth (Spore Compartment, SC).

This experiment comprised three levels of soluble Al in the growth media 1.1, 7.3 and 11.9 ppm (A_1, A_7 and A_{12}, *see* Section 2.1) placed in the SC and two levels of available P in growth media placed in the PC. There were three replicates for each treatment combination.

Growth media were a 90:10 sand and soil mixture (*see* Section 2.1) with and without addition of 36 mg P kg^{-1} soil and fertilized with Ruakura solution with the composition described in Section 2.1 except that P was omitted. The final concentrations of P (Bray 1) were 5 and 26 ppm (denoted P_5 and P_{26}). They are categorized as low and high available soil P (Olsen and Sommers, 1982). 120 g of the media were placed into each mesh bag (PC).

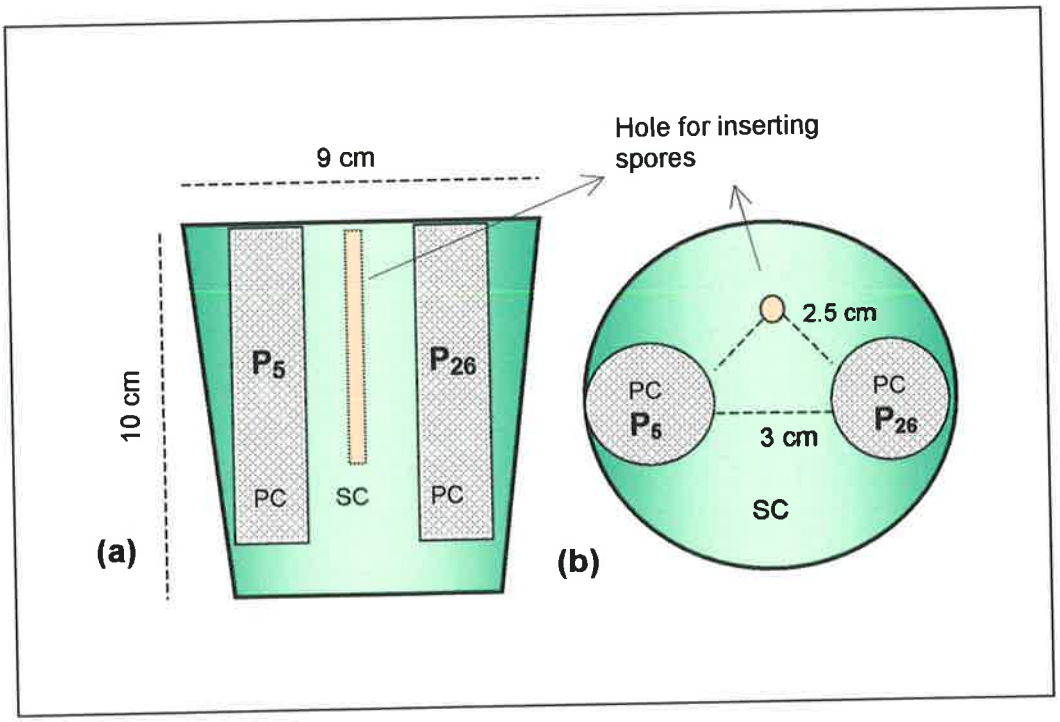

Figure 5.6 Pot system consisting of compartments for plants (PC) with different P treatments and for spores (SC) separated by 30 μm screen mesh.
(a) and (b) are views from side and above the pot.

Pre-germinated cowpea seeds were transplanted singly into each mesh bag and grown for 2 weeks before the bag was inserted into a pot (9 cm diameter and 10 cm high). There were two bags, one with and one

without P placed in every pot and arranged so that they were about 3 cm apart diametrically opposite one another (Fig. 5.6). The pot was then filled with 460 g of Al-treated growth medium. About 300 spores of *Gi. margarita* were pipetted into a hole positioned about 2.5 cm from the two bags. RO water, adjusted to pH 4.7 with H_2SO_4 was used to maintain soil moisture at field capacity by weight.

Plants were harvested 3 and 6 weeks (H_1 and H_2) after they were potted. Root colonization, shoot and root dry weights and P concentrations in shoot and roots were measured using methods described in Chapter 2.

The effect of Al on spore infectivity was determined by comparing the extent of mycorrhizal colonization in the roots of trap plants received low and high soil P treatments.

5.5.3 Results

5.5.3.1 Root colonization

Under Al stress spores of *Gi. margarita* were able to initiate root colonization. However this was considerably time-dependent. At the first harvest (H_1), the percentage of root length colonized was very low regardless of Al and P treatments but increased dramatically at H_2. It appears that the percentage of root length colonized by the fungus was affected significantly by Al but not by P. However, as can be seen in Fig. 5.7, the effect of Al was more pronounced for plants with low soil P than plants with high soil P, particularly in A_{12}.

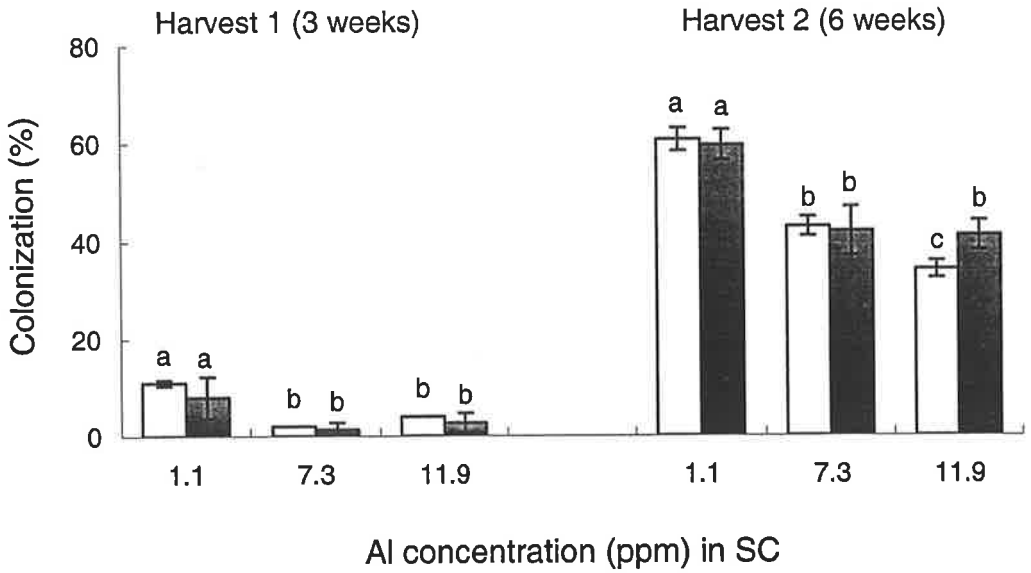

Figure 5.7 Root colonization by *Gi. margarita* on plants with low (□) and high (■) soil P availability in PC with different Al concentrations in SC at different harvest times

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

5.5.3.2 Plant growth

Overall, plants had higher dry weight (DW) at the second harvest (H_2) than at the first (H_1), though there were differences amongst them produced by the different concentrations of soil P in PC and of Al in SC.

At H_1, plants with high P grew better than those with low P irrespective of Al concentrations in SC, while at H_2 the significant effect of P was only apparent in pots with growth medium containing 11.9 ppm soluble Al. There were negative effects of Al in SC on plant growth, which increased with increasing Al concentrations in growth media. At H_2, increasing Al from 7.3 to 11.9 ppm had no effects on plants with high P but decreased the growth of plant with low P (Fig. 5.8).

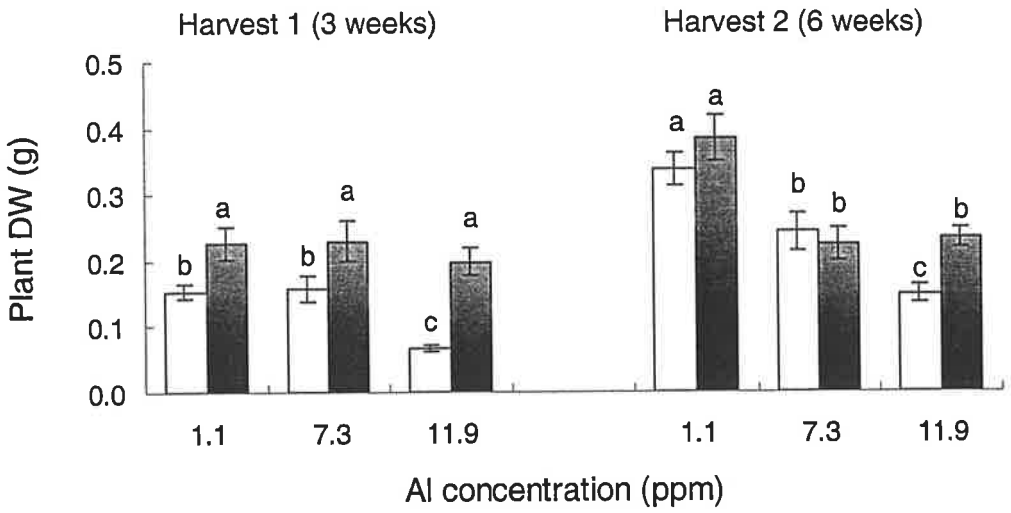

Figure 5.8 Growth of cowpea with low (□) and high (■) soil P availability in PC with different Al concentrations in SC at different harvest times.

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

5.5.3.3 Plant P concentration and uptake

Differences in soil P availability significantly affected P concentrations in shoots and roots, however the effects of the soil P varied with harvest time and also with the concentration of soluble Al (Figs. 5.9 and 5.10). Shoot or root P concentrations of plants with high soil P varied little between harvest times and with soluble Al; those of plants with low soil P, at H_1 in particular, decreased significantly with increasing Al concentrations. At H_2, however, the decreases were only observed for shoot P in A_{12}, and for root P concentrations both in A_7 and A_{12}.

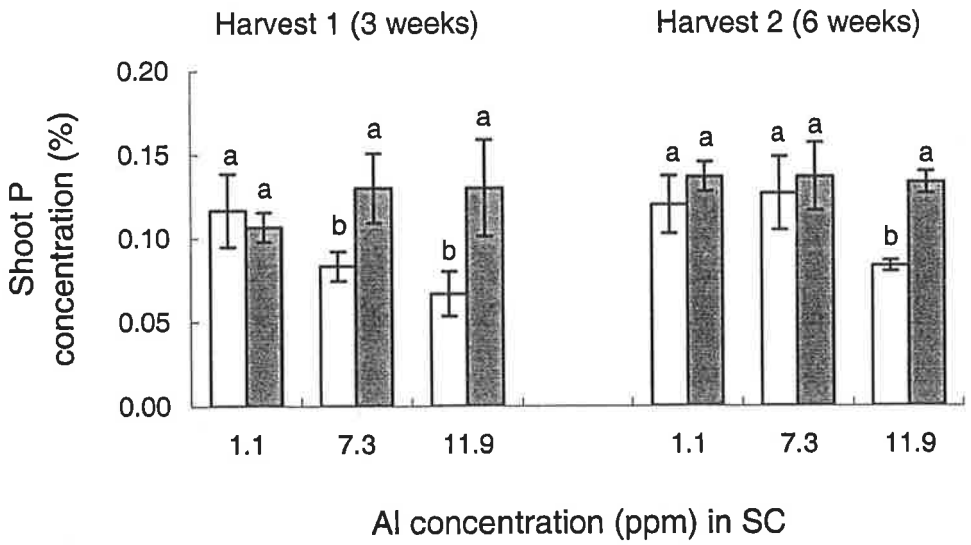

Figure 5.9 Shoot P concentrations of cowpea with low (□) and high (■) soil P availability in PC with different Al concentrations in SC at different harvest times.

Bars in the same harvest group marked with the same letters are not significantly different at P ≤ 0.05. Bars represent means and standard errors of means (n=3).

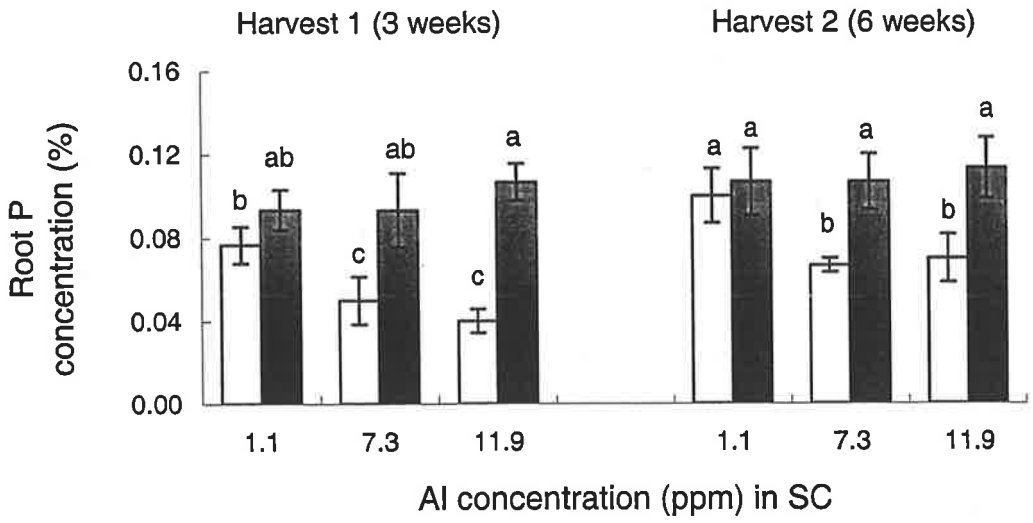

Figure 5.10 Root P concentrations of plants with low (□) and high (■) soil P availability in PC with different Al concentrations in SC at different harvest times

Bars in the same harvest group marked with the same letters are not significantly different at P ≤ 0.05. Bars represent means and standard errors of means (n=3).

Fig. 5.11 shows that plant P uptake followed a similar trend to plant growth. Plants grown with low soil P generally had lower P uptake than plants grown with high soil P particularly at H_1 and the effect of P was more distinct with increasing Al. At H_2 the effect of P addition was only significant in A_{12}.

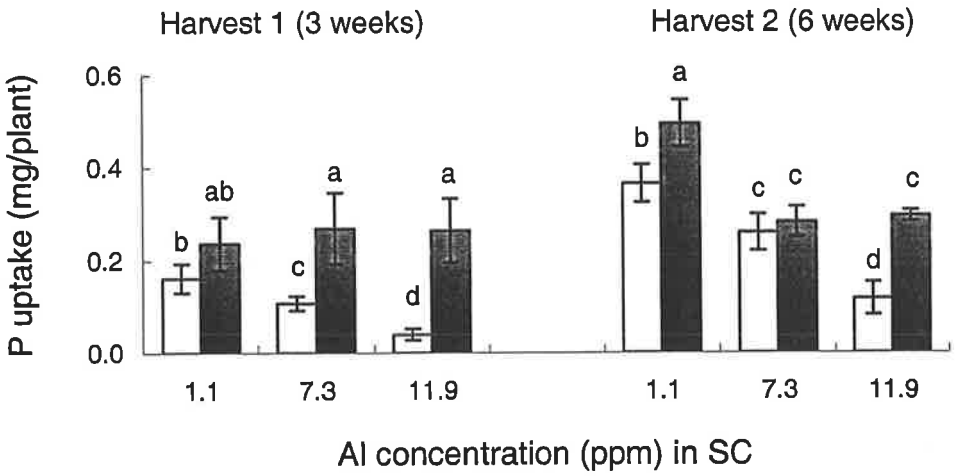

Figure 5.11 P uptake of plants with low (□) and high (■) soil P availability in PC with different Al concentrations in SC at different harvest times

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

5.6 Discussion

The results of the *in vitro* experiments (1 - 3) show that in terms of spore germination, *Gi. margarita* was tolerant to low pH as indicated by the percentage of spores germinated, *viz* 76% and 68% at pH 5.3 and 4.8 respectively, though this decreased to 40% at pH 4.5 (Fig. 5.2). This is similar to the results obtained by Siqueira *et al.* (1982). They found that *Gi. margarita* was tolerant to low pH with the best soil pH for spore germination

near 6, at which the rate of germination was 58% compared with only 48% at pH 4.5. This is also consistent with reports by Green *et al.* (1976) for other species of *Gigaspora*.

On the other hand, as shown by Figs. 5.4 and 5.5, the presence of Al in the growth medium had a negative effect on spore germination; this effect became stronger with increasing concentration. Because effects on spore germination occurred whether media with high Al contained high Na (Section 5.4) or not (Section 5.3), they must here have been due to Al and not Na. The presence of soluble Al at concentrations of 2.7 ppm in agar plates (Section 5.3), or of 7 ppm in soil (Section 5.4) significantly reduced spore germination by about 40%. This suggests that Al is an important factor inhibiting spore germination under acidic conditions, although the inhibitory effects of Al varied with the growth medium. It appears that Al toxicity was more marked in agar media than in soil. The present finding that Al inhibited spore germination in soil is not in agreement with the results of Bartolome-Esteban & Schenck (1994). Using the same technique of sandwiched spores placed in moistened acid soils differing in Al concentrations, they found that Al did not affect either spore germination or hyphal growth of *Gi. margarita*. Differences in medium conditions and fungal isolates used could be significant factors in these apparent contradictions.

The results from *in vitro* experiments on agar plates also demonstrated that both low pH and the presence of Al in growth media depressed the growth of hyphae from germinated spores. Fig. 5.3 shows that decreasing media pH from 5.3 to 4.8 and 4.5 reduced hyphal length by 34%

and 63% respectively, whereas the presence of 0.7 ppm Al in agar at pH 5.3 reduced the length of hyphae by 48%. The reduction increased as Al concentrations were increased (Fig. 5.5b). These results are contrary to another result of Siqueira *et al.* (1982). They found that germ tube growth of *Gi. margarita* was less affected than germination by low pH. The difference in growth response of hyphae to low pH in the two experiments is probably caused by different growth medium conditions. In the experiment by Siqueira *et al.* (1982), a number of mineral nutrients and thiamine-HCl had been added to the agar to stimulate hyphal growth, whilst nothing was added in the present experiments.

Inhibition of spore germination and more particularly of hyphal growth under acidic conditions may have ecological implications for successive stages in the root colonization processes. Therefore, to some extent these results support the hypothesis that a low percentage of mycorrhizal colonization by some species of AM fungi in acid soils is associated with depression of the early hyphal growth of the fungi (Robson and Abbott, 1989).

Interestingly as shown by Experiment 4 (*see* Section 5.5), under soluble Al stress spores of the fungus were able to initiate and colonize plant roots successfully within three weeks after inoculation and the percentage colonization increased over time irrespective of P treatments to trap plants (Fig. 5.7). Apparently, spores of the fungus could germinate and grow their hyphae sufficiently to contact and subsequently colonize plant roots. This

suggests the adverse effects of Al *in vitro* on the early growth of the fungus were not as pronounced in soil with the fungus and host plant living together.

The spore growth response to Al in Experiment 4 was possibly affected by the presence of plant roots nearby the spores. Some evidence from previous studies shows that although spore germination is apparently an independent event, the elongation and branching of germ tubes of the germinating spores may be stimulated by root exudates and volatiles from host plants (Gianinazzi-Pearson *et al.*, 1989; Nagahashi and Douds, 2000). Therefore the limited growth of germinating hyphae of the fungus found *in vitro* could be related to lack of eliciting factors in growth media rather than to just the adverse effects of low pH or toxic Al.

The stimulatory effects of root exudates may be different between plant species and genotypes (Gianinazzi-Pearson *et al.*, 1989; Nagahashi and Douds, 2000), or even the physiological status of plants (Tawaraya *et al.*, 1998). In this experiment, cowpea plants, used as trap plants for hyphae growing from spores, showed significant differences in growth and P nutrition status on account of soil P availability in PC and Al concentration in SC particularly at the first harvest (Figs. 5.8 - 5.11). However, these plants had no different stimulatory effects on spore infectivity of *Gi. margarita* in relation to soluble Al in SC. The only difference in the extent of colonization was observed at the highest Al concentration of 11.9 ppm at the second harvest (Fig. 5.7). Therefore, this is in contrast to Tawaraya *et al.* (1998), who found that root colonization by *Gi. margarita* was less stimulated by exudates released by P-sufficient than by P-deficient onion plants. Higher colonization

in PC with high soil P possibly resulted from colonization not only by hyphae inside the PC but also by those coming from another PC with low P due to lower growth of the host plant (Fig. 5.8).

Another interesting aspect of these results is the growth of the trap plants in the two PCs, those supplied with different available P. The growth of these had probably been improved by mycorrhizal symbiosis formed by the hyphae from germinated spores, at the second harvest in particular. At Harvest 1 plants supplied with high P were larger than those with low P (Fig. 5.8). By Harvest 2 there was no significant difference in growth between the two P treatments, except at the highest Al concentration. Good growth of the low P plants was most probably due to uptake of P from the high P compartment via the hyphae (see Fig. 5.11).

In conclusion, the experiments described in this chapter have demonstrated clearly that the two early stages of the fungal growth have different responses to acidity factors in terms of low pH *per se* and of excessive Al ions, in that the hyphae are more sensitive and so more inhibited both by low pH and Al toxicity than are spores. However, the hyphae growing from germinating spores continued to grow even under acidic soil stress with possible stimulus coming from the presence of plant roots adjacent to them. It was not evident that differences in plant growth and P nutritional status produced different stimulatory effects on the hyphae.

The role of high Na as a possible causative factor in the results presented in Section 5.5 needs to be addressed by further experimentation.

CHAPTER 6

EXTERNAL HYPHAL GROWTH AND FUNCTION OF *Gigaspora margarita* UNDER ALUMINIUM STRESS

6.1 Introduction

Hyphae of arbuscular mycorrhizal (AM) fungi growing out from colonized roots are considered to have vital functions as an extension of the root system. Therefore, mycorrhizal effectiveness is much dependent on their development in soil (Jakobsen *et al.*, 1992b).

AM fungal species differ in their ability to produce and spread hyphae in soil (Abbott and Robson, 1985; Jakobsen *et al.*, 1992a), and so may have different contributions to nutrient uptake and plant growth (Smith *et al.*, 2000). A range of soil factors may affect the hyphae (Jennings, 1995; Dix and Webster, 1995). Consequently, the development of hyphal networks and their functions can also vary considerably with soil conditions.

Results of a previous experiment described in Chapter 4, carried out by growing plants with and without mycorrhiza in conventional non-compartmented pots where plant roots and external hyphae are growing together, show that root colonization by *Gi. margarita* and subsequently its effects on cowpea plant growth decreased with increasing soluble Al concentrations. It is supposed that external hyphae of the fungus were inhibited under the experimental conditions, but a question remains since the

hyphae were not measured. There appears to be at least two likely factors inhibiting hyphal growth. First, excess Al applied to plant growth media might be toxic to the hyphae. Second, host plant growth, depressed by Al, might provide fewer infection sites or be less capable of supplying organic carbon to the fungus. However, due to practical difficulties of proper assessment using conventional pots, these two factors were the subjects of further investigations using compartmented pot systems, by which the effects of either Al toxicity or of plant nutritional status on the hyphae can be assessed separately.

This chapter presents the results of two experiments carried out to investigate the effects of excess Al in the growth medium on the growth and function of external hyphae of *Gi. margarita* in cowpea plant growth.

6.2 Experiment 1 Effect of Al on the function of external hyphae in plant growth

6.2.1 Materials and methods

This experiment was conducted under glasshouse conditions during September and October 2001.

Plastic pots divided into two compartments for plants (PC) and for fungal hyphae (HC) were used as shown in Fig. 6.1. This system enables different arrangement of soil treatments for plant roots and external fungal hyphae. The PC was a cylindrical bag of 30 μm screen mesh (3 cm diameter,

8 cm high). This mesh retains the roots but allows fungal hyphae to pass. It was placed centrally in the pot surrounded by the compartment for hyphae.

The main idea of using the compartmented pot system was to limit the access of plant roots to soil resources in PC and at the same time maximize the function of external fungal hyphae by allowing them to develop in HC and make contributions to the plant growth.

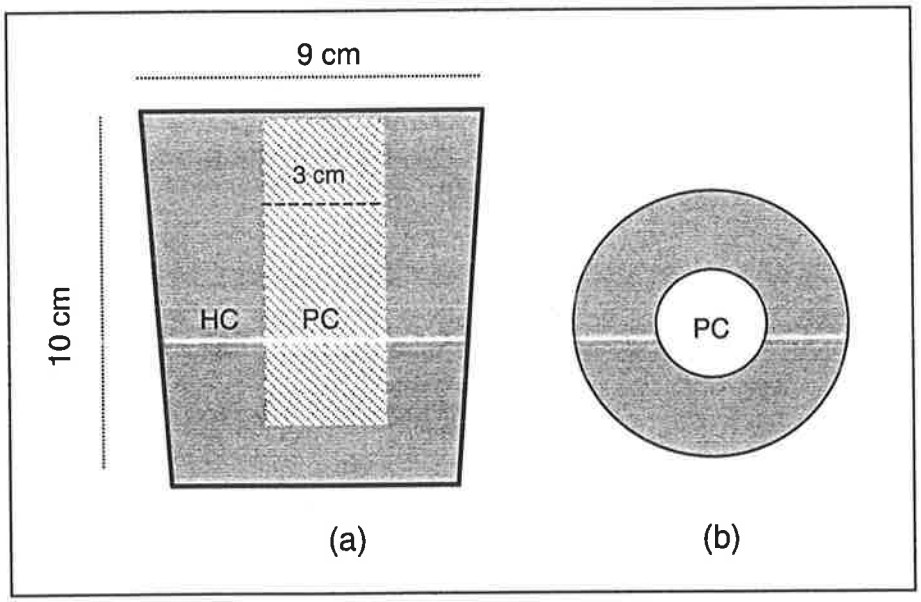

Figure 6.1 Schematic of pot system with two compartments for plant roots (PC) and external fungal hyphae (HC).
(a) and (b) are views from side and above the pot

This experiment was arranged as a factorial experiment consisting of four levels of soluble Al concentrations (1.1, 4.1, 7.3 and 11.9 ppm in the growth media of A_1, A_4, A_7 and A_{12}) placed in HC, and two levels of inoculation (with and without mycorrhiza) placed in PC, with 4 replicates. PC was filled with 120 g of no-Al treated growth medium Mo (pH 5.3; containing 0.4 ppm soluble Al and 26 ppm available P) together with 10% (w/w) pot culture inoculum with or without AM fungal structures of *Gi. margarita* (*see*

Sections 2.3 and 2.4). Therefore, plant roots and external hyphae of the fungus were grown in different soil conditions in terms of soil pH and concentrations of soluble aluminium.

Pre-germinated seeds were grown singly in PC for 2 weeks before the plant with its bag was inserted into the pot. This was to produce healthy young plants having similar growth (by visual observation only). HC was filled with 580 g of Al-treated growth medium.

RO water, adjusted to pH 4.7 with H_2SO_4, was used to maintain soil moisture at field capacity (0.1 g g^{-1} soil) and medium pH at 4.7 in HC during the experiment. After growing in the pots for 6 weeks, plants were harvested by separating shoots from roots. Representative samples of growth medium in HC were collected for assessment of external hyphal length.

Materials used in this experiment included the cowpea cultivar, the AM fungus of *Gi. margarita* and plant growth media (Mo, A_1, A_4, A_7 and A_{12}). Methods to determine shoot and root dry weights, element concentrations in plant tissues, root colonization and external hyphal length, and to analyze data are as described in Chapter 2.

In this experiment, an assumption was made that the differences in parameters measured between mycorrhizal and non-mycorrhizal plants in PC at every level of Al treatment came from differences in the function of external hyphae developing in HC. The contributions of the hyphae were determined using the formula for mycorrhizal plant responses described in Section 2.10.

6.2.2 Results

6.2.2.1 Plant growth

In general increased Al concentrations in HC had no significant effect on non-mycorrhizal plants, which is what would be expected if no soluble Al moved to the PC. However, reduced growth of mycorrhizal plants in PC, particularly shoot dry weight was observed, as shown in Table 6.1.

Table 6.1 Growth of non-mycorrhizal and mycorrhizal cowpea in PC in relation to Al concentrations in HC

Al in HC (ppm)	Mycorrhizal inoculation in PC	Dry weights of Shoots (mg)	Roots (mg)	Plant (mg)	Shoot/ root ratio	Root length (m)	MGR (%)
1.1	NM	78^{bc}	35^{a}	113^{ab}	2.1^{bc}	2.43^{ab}	0
	M	134^{a}	40^{a}	174^{a}	3.3^{a}	2.99^{a}	60
4.1	NM	85^{bc}	35^{a}	120^{ab}	2.5^{ab}	2.23^{ab}	0
	M	99^{ab}	37^{a}	136^{ab}	2.8^{ab}	2.35^{ab}	15
7.1	NM	83^{bc}	40^{a}	124^{ab}	2.1^{bc}	1.89^{b}	0
	M	80^{bc}	35^{a}	115^{ab}	2.3^{bc}	1.62^{b}	(-7)
11.9	NM	59^{c}	29^{a}	88^{b}	1.9^{c}	1.79^{b}	0
	M	52^{c}	29^{a}	81^{b}	2.0^{c}	1.68^{b}	(-8)

Figures in the same column followed by the same superscripts are not significantly different at P≤0.05; M (mycorrhiza); NM (non-mycorrhiza)
Mycorrhizal growth response (%MGR) = [{Plant $DW_{(M)}$ – Plant $DW_{(NM)}$} / Plant $DW_{(NM)}$] x 100

The table also shows that mycorrhizal inoculation had no significant effect on plant dry weight in relation to Al concentration, except shoot dry weight in the lowest soluble Al concentration at 1.1 ppm. A similar trend was

also observed for root length and shoot/root dry weight ratios. Basically, increasing Al reduced mycorrhizal growth response.

6.2.2.2 Root colonization

The concentration of soluble Al in HC did not affect percentage plant root colonization in PC (Fig. 6.2) but reduced mycorrhizal root length density (expressed in cm g^{-1} soil) (Fig. 6.3) in line with observed reductions in root growth. No colonization was observed in non-mycorrhizal control plants.

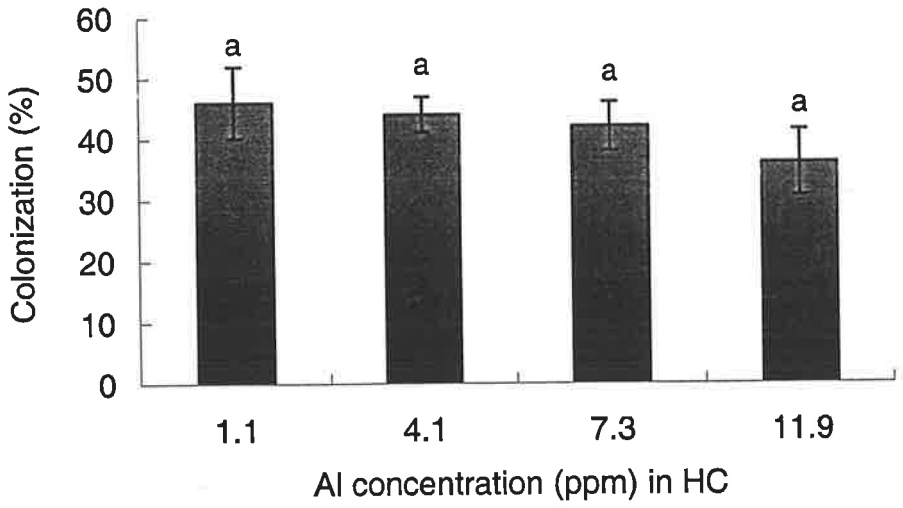

Figure 6.2 Root colonization of *Gi. margarita* in relation to Al concentrations in HC

Bars marked with the same letters are not significantly different at $P \leq 0.05$.
Bars represent means and standard errors of means (n=4)

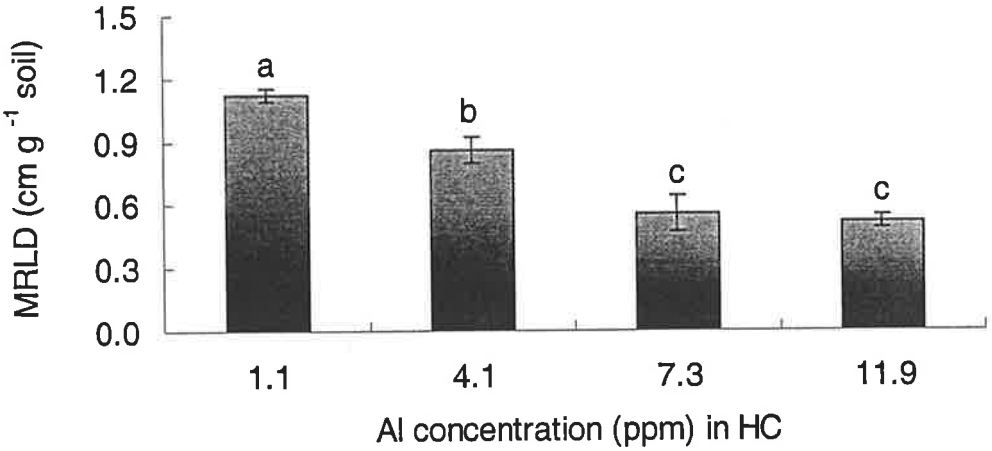

Figure 6.3 Mycorrhizal root length density (MRLD) of *Gi. margarita* in relation to Al concentrations in HC

Bars marked with the same letters are not significantly different at $P \leq 0.05$.
Bars represent means and standard errors of means (n=4)

6.2.2.3 External hyphal length in HC

After being corrected for values in control pots at the same Al treatment, the length density of external hyphae in HC, expressed in m g^{-1} soil, decreased greatly with increasing soluble Al concentrations. Compared to that in the growth medium with 1.1 ppm soluble Al, length density of the hyphae was decreased by about 50, 70 and 65% with increasing Al concentration to 4.1, 7.3 and 11.9 ppm respectively (Fig. 6.4).

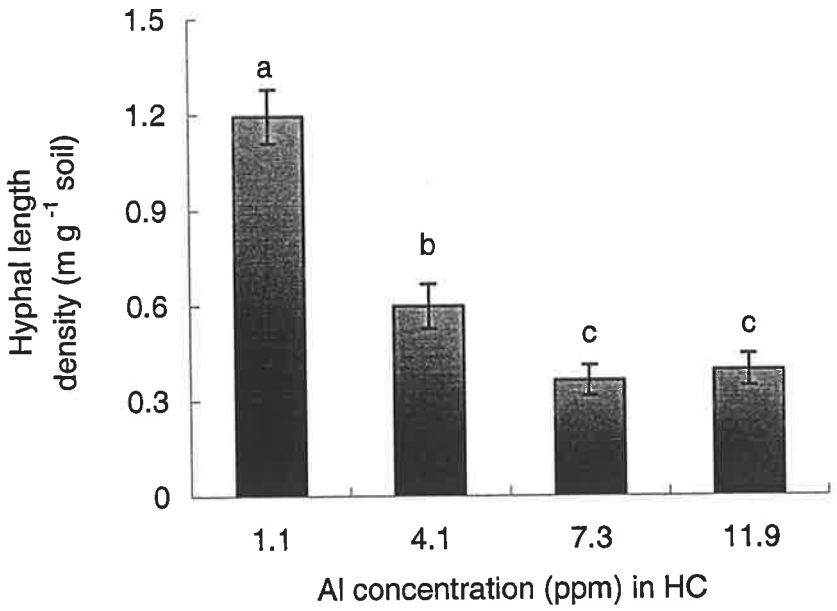

Figure 6.4 Hyphal length density of *Gi. margarita* with different Al concentrations in HC

Bars marked with the same letters are not significantly different at $P \leq 0.05$.
Bars represent means and standard errors of means (n=4)

6.2.2.4 Element concentrations in plant tissues

Element concentrations in shoot and root tissues of non-mycorrhizal and mycorrhizal cowpea plants in PC in relation to different soluble Al concentrations in HC are shown in Tables 6.2 and 6.3.

In general, concentrations of elements both in shoot and root tissues did not change significantly due to mycorrhizal inoculation. Significant increases were only observed for root P at 1.1 ppm. On the other hand, significant decreases were observed for shoot Mg, Zn and Mn at 1.1 ppm soluble Al.

Table 6.2 Element concentrations in shoot tissues of mycorrhizal and non-mycorrhizal cowpea plants in PC in relation to Al-concentrations in HC

Al in HC (ppm)	Mycorrhizal inoculation in PC	Element concentration									
		P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
		%	%	%	%	%	mg/g	mg/g	mg/g	%	mg/g
1.1	NM	0.18^{a}	1.06^{a}	0.46^{a}	4.17^{a}	0.49^{c}	0.21^{b}	0.15^{a}	0.26^{a}	0.21^{d}	0.30^{a}
	M	0.20^{a}	1.02^{a}	0.33^{b}	3.83^{a}	0.47^{c}	0.18^{b}	0.09^{b}	0.19^{b}	0.12^{d}	0.24^{a}
4.1	NM	0.19^{a}	0.78^{a}	0.31^{b}	4.03^{a}	0.52^{bc}	0.18^{b}	0.15^{ab}	0.21^{b}	0.79^{c}	0.30^{a}
	M	0.20^{a}	0.84^{a}	0.36^{b}	4.17^{a}	0.63^{b}	0.21^{b}	0.11^{ab}	0.20^{b}	0.73^{c}	0.30^{a}
7.3	NM	0.20^{a}	0.85^{a}	0.33^{b}	3.97^{a}	0.85^{a}	0.22^{b}	0.11^{ab}	0.17^{bc}	1.30^{b}	0.28^{a}
	M	0.20^{a}	0.73^{a}	0.32^{b}	4.18^{a}	0.83^{a}	0.26^{ab}	0.11^{ab}	0.16^{bc}	1.40^{b}	0.24^{a}
11.9	NM	0.22^{a}	0.52^{a}	0.32^{b}	2.62^{b}	0.94^{a}	0.31^{a}	0.08^{b}	0.13^{c}	2.59^{a}	0.43^{a}
	M	0.22^{a}	0.59^{a}	0.32^{b}	2.71^{b}	0.91^{a}	0.32^{a}	0.08^{b}	0.14^{c}	2.84^{a}	0.37^{a}

Figures in the same column followed by the same superscripts are not significantly different based at $P \leq 0.05$;
NM (non-mycorrhiza) and M (mycorrhiza).

Table 6.3 Element concentrations in root tissues of mycorrhizal and non-mycorrhizal cowpea plants in PC in relation to Al-concentrations in HC

Al in HC (ppm)	Mycorrhizal inoculation in PC	Element concentration									
		P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
		%	%	%	%	%	mg/g	mg/g	mg/g	%	mg/g
1.1	NM	0.12^{b}	0.37^{a}	0.17^{b}	5.53^{a}	0.75^{b}	0.81^{a}	0.13^{a}	0.03^{a}	0.25^{c}	2.66^{b}
	M	0.21^{a}	0.39^{a}	0.21^{ab}	6.00^{a}	0.82^{ab}	0.54^{a}	0.13^{a}	0.02^{a}	0.33^{c}	2.76^{b}
4.1	NM	0.15^{b}	0.29^{a}	0.19^{b}	5.51^{a}	1.02^{a}	0.48^{a}	0.13^{a}	0.03^{a}	0.60^{b}	3.04^{ab}
	M	0.17^{ab}	0.31^{a}	0.19^{b}	4.89^{ab}	0.95^{ab}	0.52^{a}	0.11^{a}	0.03^{a}	0.61^{b}	2.69^{ab}
7.3	NM	0.16^{ab}	0.32^{a}	0.19^{b}	4.95^{ab}	0.94^{ab}	0.74^{a}	0.13^{a}	0.02^{a}	0.66^{b}	3.94^{a}
	M	0.14^{ab}	0.27^{a}	0.17^{b}	3.21^{b}	0.73^{b}	0.74^{a}	0.12^{a}	0.02^{a}	0.59^{b}	3.50^{ab}
11.9	NM	0.16^{ab}	0.26^{a}	0.20^{ab}	3.55^{b}	0.98^{ab}	0.45^{a}	0.11^{a}	0.03^{a}	1.51^{a}	1.93^{b}
	M	0.15^{b}	0.28^{a}	0.26^{a}	3.88^{b}	1.14^{a}	0.53^{a}	0.11^{a}	0.02^{a}	1.89^{a}	2.17^{b}

Figures in the same column followed by the same superscripts are not significantly different based at $P \leq 0.05$;
NM (non-mycorrhiza) and M (mycorrhiza).

6.2.2.5 Element uptake

Total uptake of some selected elements by plants in relation to mycorrhizal inoculation and Al treatments is shown in Table 6.4.

It appears that increased Al had no effect on S, Zn and Fe but increased Na and generally reduced P, Ca, Mg, K and Mn uptake particularly at the highest Al concentration (11.9 ppm). The uptake of Al particularly by non-mycorrhizal plants increased with increasing Al up to 7.3 ppm, but this then decreased with a further Al increase. The increased Na concentration in tissues of non-mycorrhizal and mycorrhizal plants with increasing Al in the HC may be due to movement of Na to the PC.

In general, as shown by mycorrhizal element uptake responses in Table 6.5, mycorrhiza made no significant contribution to improving plant element uptake under Al stress conditions in HC. The only significant increases were for P and Ca uptake in the lowest soluble Al concentration at 1.1 ppm. On the other hand, increasing Al concentration to 7.3 ppm or higher decreased the element uptake response by plants to mycorrhiza and this occurred for all elements measured.

Table 6.4 Total element uptake by mycorrhizal and non-mycorrhizal cowpea plants in PC in relation to Al-concentrations in HC

Al in HC (ppm)	Mycorrhizal inoculation in PC	Element uptake (mg)									
		P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
1.1	NM	0.18^{b}	0.96^{b}	0.39^{ab}	5.05^{ab}	0.66^{a}	0.044^{a}	0.015^{a}	0.019^{ab}	0.23^{c}	0.106^{b}
	M	0.35^{a}	1.52^{a}	0.53^{a}	7.47^{a}	0.97^{a}	0.046^{a}	0.017^{a}	0.027^{a}	0.30^{c}	0.143^{ab}
4.1	NM	0.21^{b}	0.76^{bc}	0.31^{ab}	5.16^{ab}	0.77^{a}	0.032^{a}	0.017^{a}	0.018^{ab}	0.84^{b}	0.128^{ab}
	M	0.25^{ab}	0.95^{b}	0.43^{ab}	5.88^{ab}	0.96^{a}	0.038^{a}	0.016^{a}	0.021^{a}	0.91^{b}	0.131^{ab}
7.3	NM	0.23^{ab}	0.82^{bc}	0.35^{ab}	5.22^{ab}	1.08^{a}	0.050^{a}	0.014^{a}	0.015^{ab}	1.36^{b}	0.182^{a}
	M	0.21^{b}	0.67^{bc}	0.31^{ab}	4.52^{ab}	0.91^{a}	0.048^{a}	0.013^{a}	0.014^{b}	1.35^{b}	0.141^{ab}
11.9	NM	0.18^{b}	0.38^{c}	0.25^{b}	2.64^{b}	0.86^{a}	0.032^{a}	0.008^{a}	0.008^{b}	2.01^{a}	0.083^{b}
	M	0.16^{b}	0.38^{c}	0.24^{b}	2.51^{b}	0.80^{a}	0.031^{a}	0.008^{a}	0.008^{b}	1.95^{a}	0.079^{b}

Figures in the same column followed by the same superscripts are not significantly different based at $P \leq 0.05$;
NM (non-mycorrhiza) and M (mycorrhiza).

Table 6.5 Plant element uptake responses to mycorrhiza in PC in relation to Al-concentrations in HC

Al in HC (ppm)	Element uptake (% response)									
	P	Ca	Mg	K	S	Fe	Zn	Mn	Na	Al
1.1	92*	58*	35	48	46	4	13	41	32	35
4.1	17	24	36	14	25	19	(-5)	16	8	2
7.3	(-9)	(-19)	(-11)	(-14)	(-16)	(-4)	(-7)	(-7)	(-1)	(-22)
11.9	(-11)	(-2)	(-3)	(-5)	(-7)	(-2)	(-6)	(-6)	(-3)	(-5)

Mycorrhizal element uptake response (%) = [{$Uptake_{(M)}$-$Uptake_{(NM)}$} / $Uptake_{(NM)}$] x 100
NM (non-mycorrhiza) and M (mycorrhiza); Values marked with * are based on data values significantly different from each other in Table 6.5

6.3 Experiment 2 Effect of Al on the growth of external hyphae

6.3.1 Material and methods

This experiment was designed to investigate the effect of Al on the growth of external hyphae and their function in terms of initiating new colonization in particular.

PVC pots were used and were divided into three compartments by two vertical 30 μm screen meshes that can be passed by hyphae but exclude roots (*see* Fig. 6.8). The first compartment, Donor Plant Compartment (DPC), was for plants inoculated with or without mycorrhiza fungal structures, which

acted as donor plants. The second, Hyphal Compartment (HC), in the middle pot, was a root free compartment provided for external fungal hyphae extending from DPC to grow alone. The third, Receiver Plant Compartment (RPC), was for uninoculated plants (as receiver plants), which acted as trap plants to be colonized later by hyphae traversing HC from donor plants. Both plant compartments DPC and RPC were filled with no Al-treated growth medium Mo (pH 5.3, containing 0.4 ppm soluble Al and 26 ppm available P; *see* Section 2.1), in order to provide favourable conditions for plant roots. HC was filled with Al-treated growth media differing in soluble Al concentrations (*see* below).

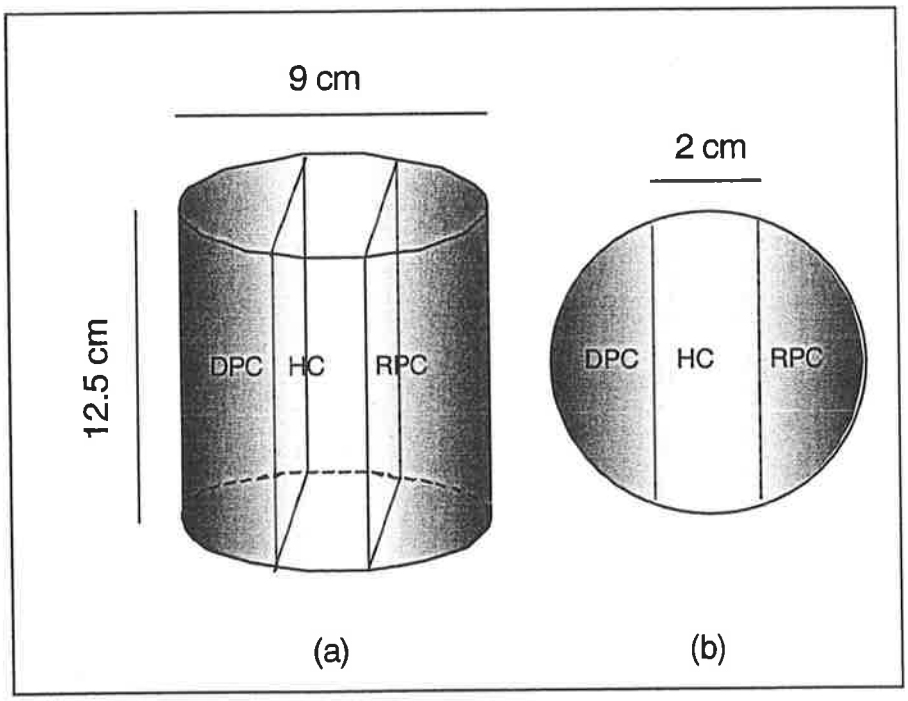

Figure 6.5 Schematic of pot system with three compartments for roots of donor (DPC) and receiver (RPC) plants and for external fungal hyphae (HC)
(a) and (b) are views from side and above the pot.

The idea of this experiment was to let external hyphae from DPC grow into HC, interact with conditions of Al stress and if possible to traverse the compartment to RPC, and to colonize roots of trap plants there. Therefore, the effects of Al in HC on the growth of external hyphae were indicated by length density of the hyphae in the HC and/or the extent of root colonization of trap plants in RPC.

This experiment was conducted under glasshouse conditions in September - November 2001. It was a completely randomized design consisting of three levels of soluble Al concentrations (1.1, 4.1, and 7.3 ppm in growth media A_1, A_4 and A_7) placed in HC and two levels of inoculation (with and without mycorrhiza) placed in DPC, with three replicates.

Materials used for this experiment including the cowpea cultivar, *Gi. margarita* isolate, and growth media (M_O, A_1, A_4 and A_7) were the same as used in the first experiment Section 6.2, except growth medium A_{12} that contained the highest concentration of soluble Al (11.9 ppm). This growth medium was omitted in this experiment because of its similar effect on hyphal growth to that of A_7 (*see* Fig. 6.5).

At beginning of the experiment, DPC and RPC were filled with 320 g of growth medium M_O (containing 26 ppm available P, 0.4 ppm soluble Al, pH 5.3), but only DPC was inoculated with 10% (w/w) of pot culture inoculum with or without fungal structures to provide mycorrhizal and non-mycorrhizal plants. Pre-germinated seeds of cowpea were transplanted into both DPC and RPC, 2 plants in each compartment, and grown for two weeks whilst HC was left empty. HC was then filled with 260 g of Al-treated growth media A_1,

A_4 or A_7. RO water, adjusted to pH 4.7 with H_2SO_4, was used to maintain the moisture content of growth media at field capacity (about 0.1 g g^{-1} soil) and media pH at about 4.7, particularly in HC, throughout the experiment.

Plants were harvested 4 and 8 weeks (H_1 and H_2) after HC was filled. Dry weights, P uptake and mycorrhizal colonisation of roots for both donor and receiver plants and the length of external fungal hyphae in HC were measured using procedures described in Chapter 2.

Data were analyzed statistically using ANOVA after grouping into plant compartments and harvest times. LSD tests at $P \leq 0.05$ were then used to determine differences amongst the means of treatments.

6.3.2 Results

6.3.2.1 Donor plant growth and P uptake

The effects of Al treatments in HC and mycorrhizal inoculation on the growth of plants in DPC at different harvest times are presented in Fig. 6.6. At H_1, the growth of mycorrhizal donor plants in DPC was unaffected by Al treatments in HC, while that of control plants with no mycorrhizal inoculation decreased slightly with increasing Al concentration in HC. Dry weight of mycorrhizal plants was higher than that of control plants and to a greater extent at higher Al concentrations. At H_2, increased Al concentrations slightly increased both the growth of mycorrhizal and non-mycorrhizal plants in DPC. Furthermore, regardless of Al treatments, mycorrhizal colonization had a dramatic stimulatory effect on the growth of donor plants.

Fig. 6.7 shows that root lengths of plants in DPC did not change with Al concentrations, but increased between 4 and 8 weeks. Mycorrhizal inoculation generally increased plant root length, though a significant increase only occurred in A_7 at H_1 or H_2.

The effects of Al treatments and mycorrhizal inoculation on plant P uptake show a similar trend to plant growth (Fig. 6.8). The amount of P taken up by mycorrhizal and non-mycorrhizal plants increased over time. Al treatments had no significant effects on P uptake by the two plants irrespective of harvest times, but mycorrhizal plants took up more P than control plants, primarily at H_2.

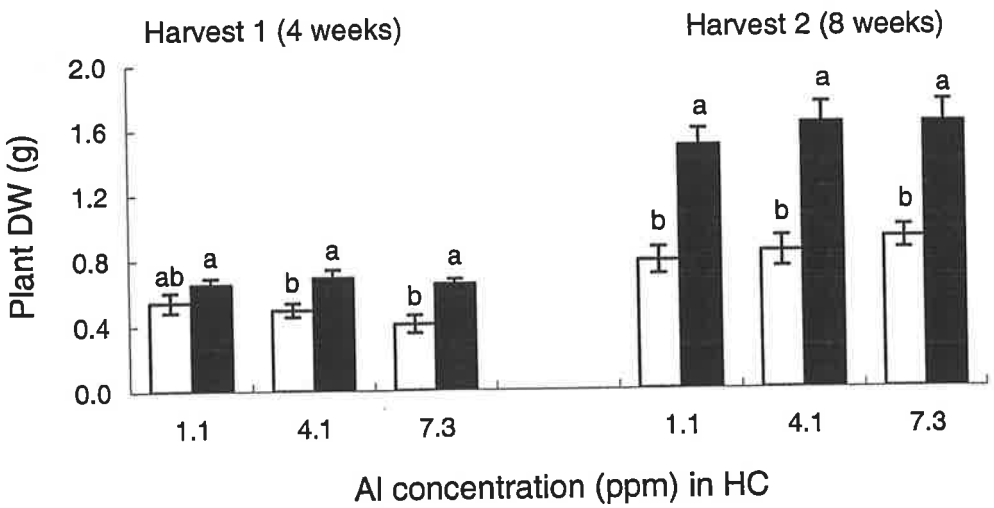

Figure 6.6 Dry weights of non-mycorrhizal (□) and mycorrhizal (■) plants in DPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

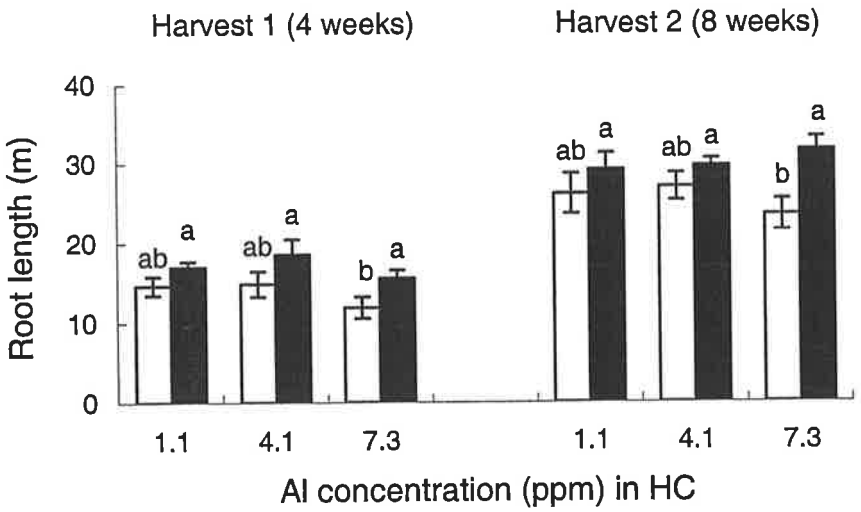

Figure 6.7 Root lengths of non-mycorrhizal (□) and mycorrhizal (■) plants in DPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

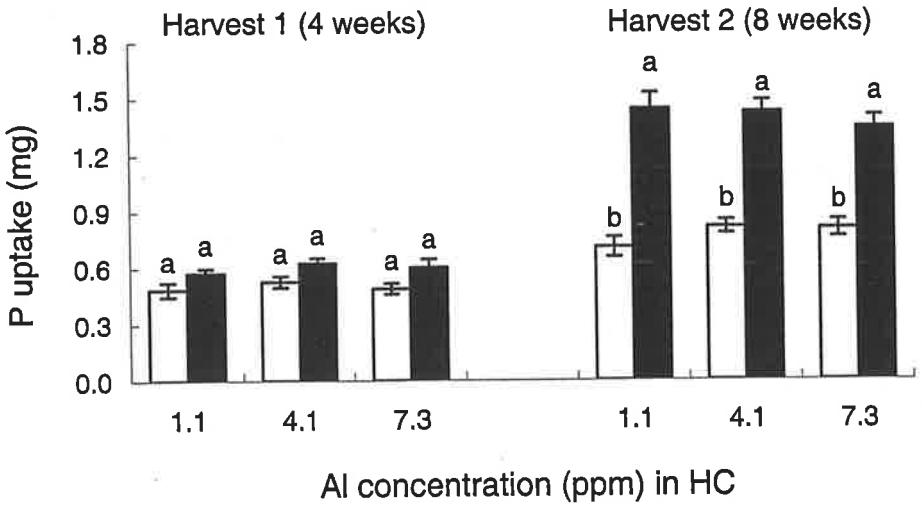

Figure 6.8 P uptake by non-mycorrhizal (□) and mycorrhizal (■) plants in DPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

6.3.2.2 Root colonization of donor plants

The percentages of root length colonized by *Gi. margarita* in DPC in relation to the effects of Al treatments given in HC are shown in Figs. 6.9. No colonization was observed in un-inoculated mycorrhizal plants. Root colonization of donor plants either at H_1 or H_2 did not change with Al concentrations in HC, but increased with harvest time. Root colonization at H_2 was higher (about 70%) than at H_1 (45%).

Mycorrhizal root length density (MRLD) of donor plants showed a similar trend to percentage colonization as expected (Fig. 6.10), because treatment had no effect on root length (Fig. 6.7).

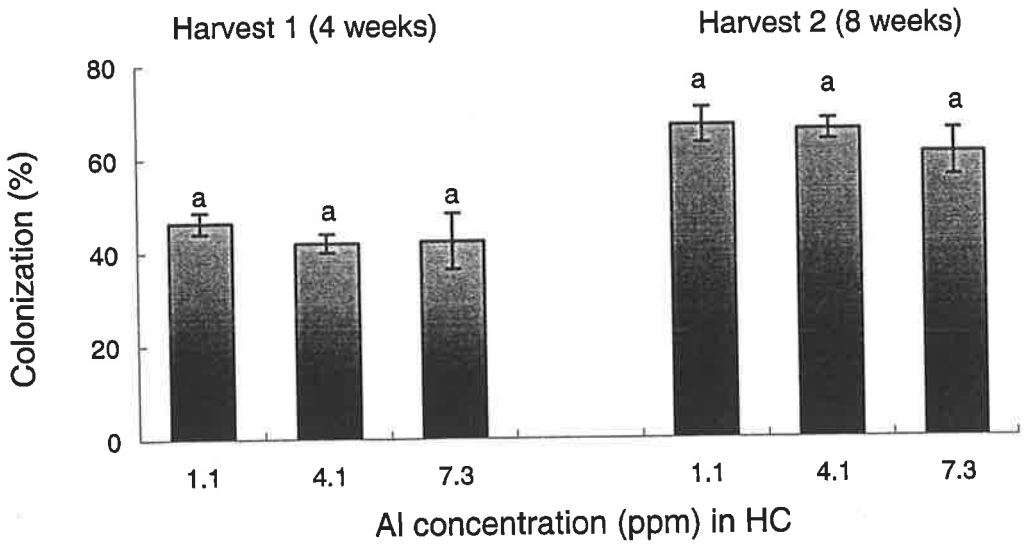

Figure 6.9 Root colonization of donor plants in DPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

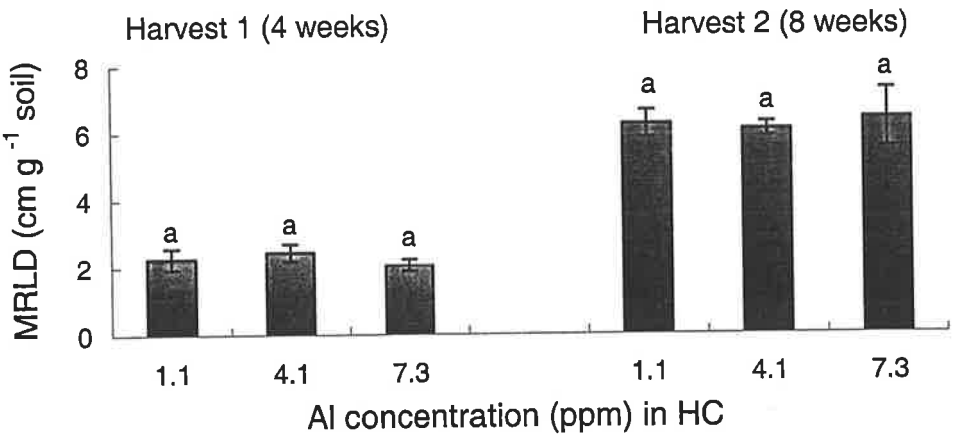

Figure 6.10 Mycorrhizal root length density (MRLD) of donor plants in DPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

6.3.2.3 External hyphal growth in HC

The growth of external fungal hyphae in HC was time-dependent and to some extent, negatively affected by increased Al concentrations (Fig. 6.11).

At H_1, length density of the hyphae at 1.1 ppm soluble Al was not different from that at 4.1 ppm but significantly higher than that at 7.3 ppm; these were 3.9, 3.5 and 0.9 m g^{-1} dry soil respectively. At H_2, there was a similar trend in the growth of hyphae relating to the effect of Al treatments. However, hyphal length density at H_2 was much higher than at H_1.

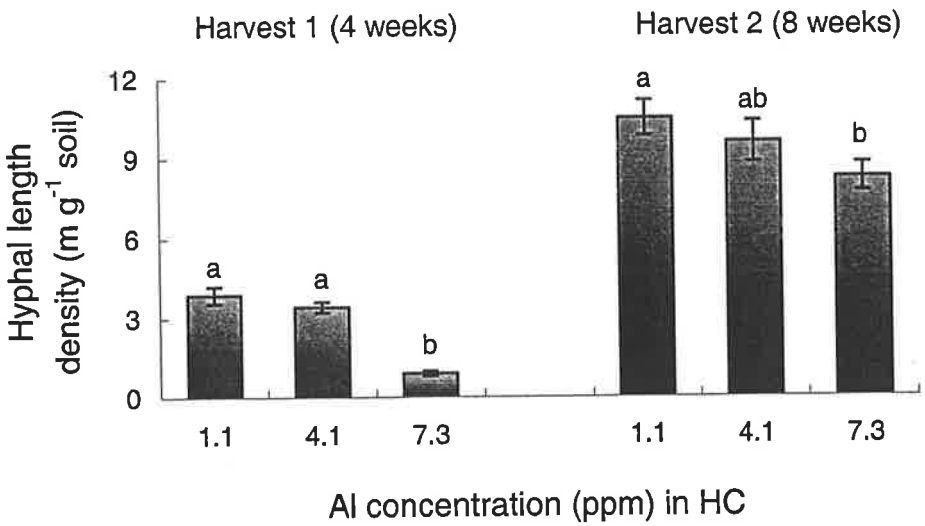

Figure 6.11 Hyphal length density in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

6.3.2.4 Root colonization of receiver plants

Both Al concentrations in HC and harvest times influenced the extent of mycorrhizal colonization of receiver plant roots. At H_1, the colonization was less than 20%, but increased to more than 40% at H_2. Different Al concentrations in HC affected the root colonization differently. Compared to that at 1.1 ppm soluble Al, a significant reduction in root colonization at H_1 had occurred when 7.3 ppm was present, and this reduction was already evident at 4.1 ppm at H_2 (Fig. 6.12).

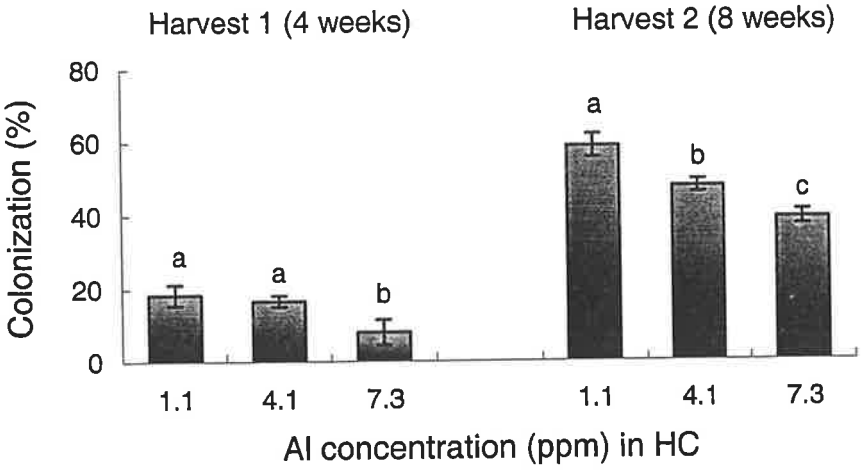

Figure 6.12 Root colonization of receiver plants in RPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

6.3.2.5 Receiver plant growth and P uptake

In general, there were no significant differences in growth of plants in RPC in relation to soluble Al concentrations in HC, but growth was generally improved by mycorrhizal colonization. Although it was not different at H_1, dry weights of mycorrhizal plants were significantly higher than that of non-mycorrhizal plants regardless of Al concentrations in HC (Fig. 6.13).

P uptake by plants in RPC shows a similar trend to plant growth (Fig. 6.14). Total P taken up by mycorrhizal and non-mycorrhizal plants increased over time. P uptake by mycorrhizal and non-mycorrhizal plants at H_1 was not different regardless of Al treatments, but a mycorrhizal advantage was evident at H_2 when increased Al concentrations in HC to 7.3 ppm reduced P uptake by non-mycorrhizal plants but not by mycorrhizal plants.

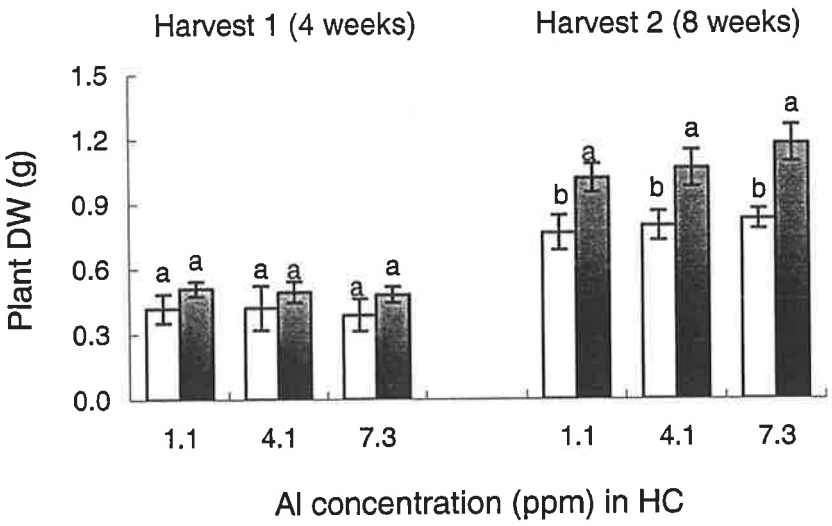

Figure 6.13 Dry weights of non-mycorrhizal (□) and mycorrhizal (■) plants in RPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

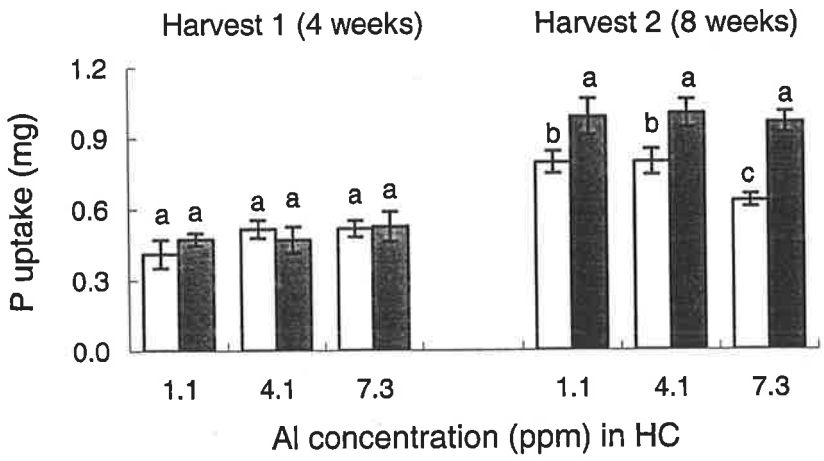

Figure 6.14 P uptake by non-mycorrhizal (□) and mycorrhizal (■) plants in RPC in relation to Al concentrations in HC

Bars in the same harvest group marked with the same letters are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3)

6.4 Discussion

Results of the first experiment using separate compartments for plant (PC) and hyphae (HC) showed in general that growth of plants in PC decreased with increasing soluble Al concentrations in HC regardless of mycorrhizal inoculation (Table 6.1). The trend was small and not significant for non-mycorrhizal plants so that movement of Al ions directly from HC to PC probably did not affect growth. The high mobility of soluble Al in soil solution under acidic conditions, as has been pointed out in some reviews (*see* Huang, 1988; McBride, 1994; Wolt, 1994), was probably not important here. However, increasing concentrations of Na in tissues of both non-mycorrhizal and mycorrhizal plants suggest that Na did move between compartments.

This experiment also showed that the major effect of mycorrhizal inoculation in benefiting plant growth was in pots with the lowest soluble Al concentration at 1.1 ppm, and the beneficial effects of the symbiosis decreased with increasing Al. Increased Al concentrations to 7.3 ppm or higher produced no or slightly negative mycorrhizal responses, in terms of plant growth and element uptake (Tables 6.1 and 6.4). If it is assumed that any difference between mycorrhizal and non-mycorrhizal plants in PC is induced by external hyphae developing in HC, these results suggest that the external hyphae of *Gi. margarita* did not function effectively in soil with high soluble Al.

Although percentage root colonization in PC was not different at different Al concentrations in HC (Fig. 4.2), the length density of external

hyphae with any soluble Al present above 1 ppm was much lower (Fig. 6.4). This indicates that the growth of hyphae in HC from colonized roots in PC was not related to the intensity of root colonization, but was negatively affected by Al concentration. For example, increasing soluble Al from 1.1 to 4.1 ppm reduced the hyphal density by about 50% (Fig. 6.4). Therefore, from this standpoint, it is possible that the deficiency of mycorrhizal contributions was a consequence of limited growth and/or distribution of fungal hyphae away from root surfaces into the growth medium (Jakobsen *et al.*, 1992a, b). Accordingly, in such a situation no increase in nutrient acquisition for the plants could be expected, as has also already been shown in Chapter 4.

Decreased responses of plants to mycorrhiza in terms of plant growth and element uptake with increasing Al concentrations (Tables 6.1 and 6.5) could probably be caused by imbalances in the benefit-cost ratio of the symbiosis for the plants (Johnson *et al.*, 1997). Plants obtained only small benefits from the symbiosis due to inhibited growth of fungal hyphae by Al but at the same time had to spend a similar amount of carbohydrate (Pfeffer *et al.*, 1999) to maintain high rates of mycorrhizal colonization (Fig. 6.2).

Interestingly, in the second experiment using three compartments (a central one for hyphal growth [HC] and two outer ones for donor [DPC] and receiver [RPC] plants respectively) it was found that external hyphae of the fungus, spreading from mycorrhizal donor plants in DPC, were able to grow extensively in HC containing different soluble Al and traverse it into RPC, and subsequently colonize receiver plants there. Growth of the hyphae in HC increased with harvest times, but the growth decreased with increasing Al

concentrations (Fig. 6.11). In general, compared to the first experiment (Fig. 6.2), the densities of the hyphae per g soil in this experiment were much higher. Apparently, despite depending greatly on harvest times, higher density of the hyphae in the second experiment, particularly at H_2, might result from higher mycorrhizal root length density (*see* Figs. 6.3 and 6.10). The difference might also be related to volume of Al-treated medium in the pots. The smaller volume of medium in the second experiment (260 g, compared to 580 g in the first experiment) would result in smaller negative effect of Al on the hyphae. Movement of Na from HC to DPC and RPC would have occurred but would have had little effect on plant growth because of the relatively large size of the latter compartments. The movement of Al would be expected to be much less. Moreover, the presence of roots of trap plants in the second experiment might contribute to the difference. Previous workers have shown the stimulatory effects of host plant roots on hyphal growth (Giovannetti *et al.*, 1993; Koske, 1982; *see* also Section 5.5 Chapter 5).

Importantly, as shown in Fig. 6.12, root colonization of receiver plants reached more than 40% at H_2 irrespective of Al concentrations in HC. This indicates that exposure to Al stress did not really affect the function of the hyphae of *Gi. margarita* as inoculum. The hyphae remained viable to initiate new colonization. In addition, Figs 6.13 and 6.14 indicate that the hyphae did not lose their functions in increasing plant growth after being exposed to Al concentrations in HC, although there was a delay in seeing a significant contribution to the plants.

In conclusion the presence of soluble Al with increasing concentrations in growth media in HC reduced the growth of external hyphae of *Gi. margarita* and subsequently their functions in plant growth and element uptake. However, the presence of neighbouring plants might trigger the external hyphae of the fungus to grow further despite Al stress in the growth medium. Under Al stress, high colonization by the fungus, coupled with a lack of external hyphal development tended to induce negative responses of the cowpea plant to mycorrhiza.

CHAPTER 7

EFFECT OF PHOSPHORUS ON THE GROWTH OF EXTERNAL HYPHAE OF *Gigaspora margarita* UNDER ALUMINIUM STRESS

7.1 Introduction

Despite important environmental stresses such as Al toxicity in acidic soils, the growth of external hyphae of arbuscular mycorrhizal fungi is supposed to be mainly affected by the host plant since the energy for fungal growth is entirely sourced from the host plant (Bago *et al.*, 2000) and in consequence any change in plant physiology induced by the environment would be likely to affect the well-being of the fungus. P supplies that increase plant growth have been shown to reduce internal colonization (Thomson *et al.*, 1991; Amijee *et al.* 1989; de Miranda *et al.*, 1989; Koide and Li, 1990; Braunberger *et al.*, 1991) and this can be expected to reduce the length density of external hyphae as well (Abbott *et al.*, 1984).

In a previous experiment (Section 6.3) it was evident that external hyphae of *Gi. margarita* were able to grow well in Al-treated growth media containing soluble Al up to 7.3 ppm, a level that is very toxic to cowpea plants (Fig. 2.1), particularly when the trap plants were present (*see* Discussion in Chapter 6). However, further verification is needed to establish whether the hyphae remain able to grow at a higher Al concentration and if the hyphal ability to tolerate excess Al was related to the growth status of host plants

that were grown in growth medium M_o with sufficient mineral nutrients, including 26 ppm available P (Bray 1) (*see* Table 2.2).

The aim of the experiment described here was to investigate whether the different soil P availability that affected the host plant growth influenced the tolerance of external hyphae of *Gi. margarita* to a high soluble Al concentration in soil. The experimental design (including only one Al level) meant that interactive effects of Al and P supply could not be evaluated.

7.2 Materials and methods

The experiment was carried out under glasshouse conditions in December 2001 - February 2002. The compartmented pot system used was the same as in Experiment 2 Section 6.3. There were three compartments separated by meshes for donor plants (DPC), external hyphae (HC) and for receiver plants (RPC) respectively.

The experiment was set up within a completely randomised design with three replications. Treatments consisted of three concentrations of available P in soil and two inoculation treatments; all were placed in DPC. For the other compartments, HC was filled with growth medium of A_{12} (*see* detail in Table 2.2) containing 11.9 ppm soluble Al, a level that killed cowpea seedlings (Chapter 4) and inhibited spore germination and hyphal growth of the fungus (Sections 5.4 and 6.2), and the RPC was filled with growth medium Mo that contained 26 ppm available P at pH 5.3.

The soil P concentrations in DPC were 5, 16 or 26 ppm available P Bray 1 at pH 5.3 (denoted P_5, P_{16} and P_{26} respectively with the subscript referring to available P concentration), categorized as low, intermediate and high levels of P availability in soil (Olsen and Sommers, 1982). These were previously set up with additions of 0, 18 and 36 mg P kg^{-1} soil using KH_2PO_4, and the modified Ruakura solution with the composition as described in Section 2.1 except that P was omitted.

At the beginning of the experiment, P-treated growth media were mixed thoroughly with 10% of pot culture inoculum with or without fungal structures (*see* Section 2.3). 320 g of the mix was then placed in DPC (Donor Plant Compartment). At the same time, RPC (Receiver Plant Compartment) was filled with 320 g of growth medium Mo for all pots without any inoculation. HC (Hyphal Compartment) was left empty.

Two pre-germinated seeds of cowpea were transplanted both into DPC (for donor plants) and RPC (for receiver plants), and grown for 2 weeks. At 2 weeks 260 g of growth medium A_{12} (containing 25 ppm available P in addition to11.9 ppm soluble Al) was placed in HC.

Procedures in this experiment for maintaining plant growth, harvesting, measurement of plant dry weight, mycorrhizal colonization, hyphal length and plant P concentration and data analysis were the same as carried out for Experiment 2 Section 6.3.

7.3 Results

7.3.1 Donor plant growth and P uptake

Growth of donor plants expressed as plant dry weight (DW) and root length was influenced by P treatment and mycorrhizal inoculation in DPC and by harvest time (Figs. 7.1 and 7.2).

At H_1, the growth of plants with low P concentration was very poor irrespective of mycorrhizal inoculation and plants without mycorrhiza died between 4 and 8 weeks. Soil P concentrations affected the growth of mycorrhizal and non-mycorrhizal plants differently. Dry weight (DW) of mycorrhizal plants increased proportionally with the concentrations of P in soil. DW of non-mycorrhizal plants increased between P_5 and P_{16} but showed no further increase with P_{26}. A significantly higher DW of mycorrhizal over non-mycorrhizal plants only occurred with P_{26}. At H_2, the growth of mycorrhizal plants had increased markedly compared with H_1 and exhibited a similar trend with P treatment. The growth of non-mycorrhizal plants was unchanged with P_{26} or smaller with P_{16} compared with equivalent plants at H_1.

Similar trends to plant growth were observed for the length of plant roots (Fig. 7.2) and also for the P uptake of mycorrhizal and non-mycorrhizal plants in DPC (Fig. 7.3).

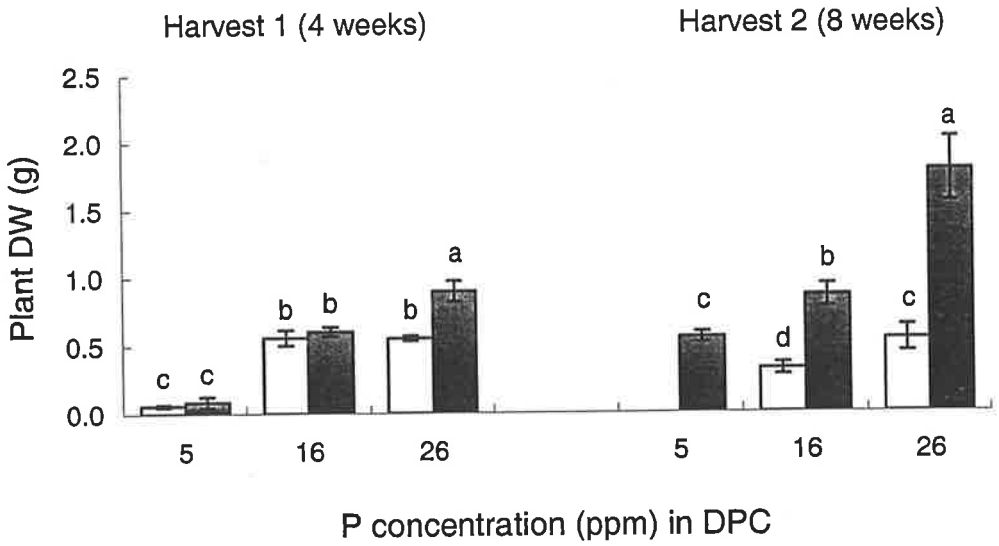

Figure 7.1 Effects of soil P concentration on plant dry weights of non-mycorrhizal (□) and mycorrhizal (■) plants in DPC at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

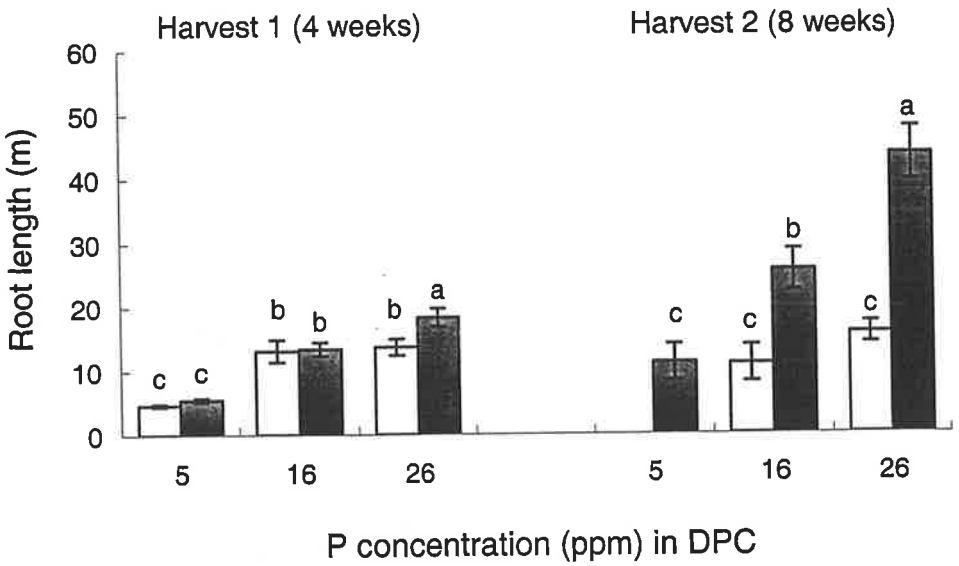

Figure 7.2 Effects of soil P concentration on plant root length of non-mycorrhizal (□) and mycorrhizal (■) plants in DPC at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

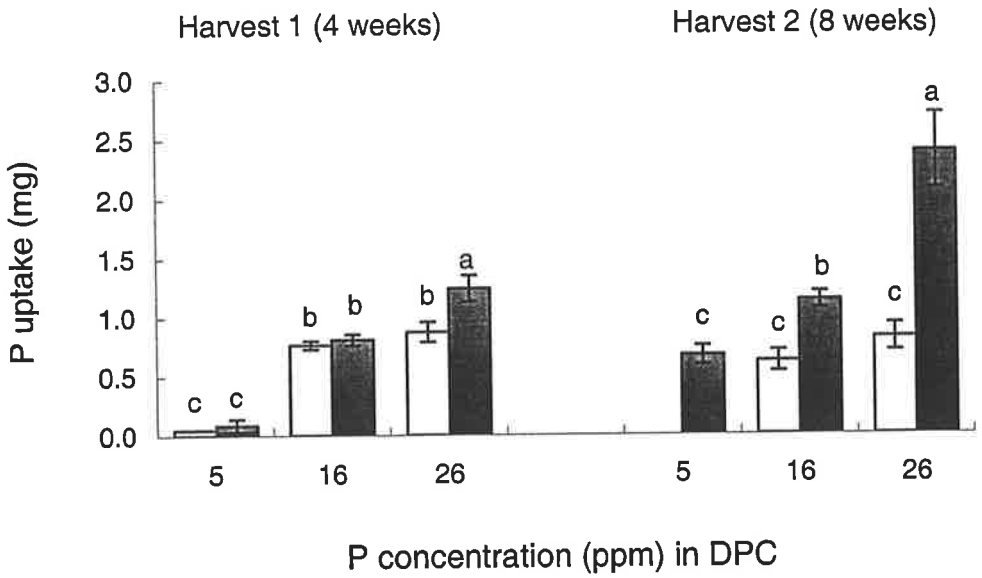

Figure 7.3 Effects of soil P concentration on P uptake of non-mycorrhizal (□) and mycorrhizal (■) plants in DPC at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

7.3.2 Root colonization of donor plants

The extent of mycorrhizal colonization of the roots of donor plants inoculated by *Gi. margarita* at two harvest times is presented in Fig. 7.4. The percentage root colonization was quite high (about 60%) and the same for all P treatments and both harvests. No colonization was observed in uninoculated donor plants.

Mycorrhizal root length density, expressed in cm g^{-1} soil, was significantly affected by P treatment irrespective of harvest times (Fig. 7.5). Increased available P in soil increased mycorrhizal root length density with greater increases at H_2.

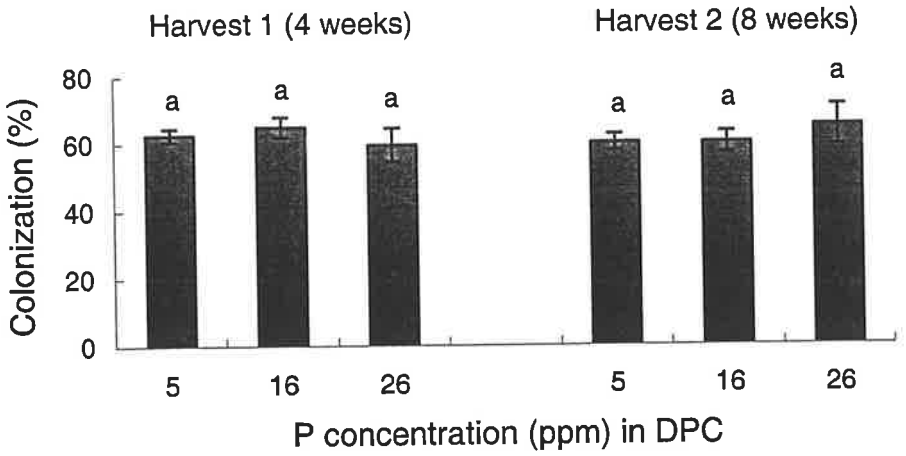

Figure 7.4 Effects of soil P concentration in DPC on root colonization of mycorrhizal donor plants at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

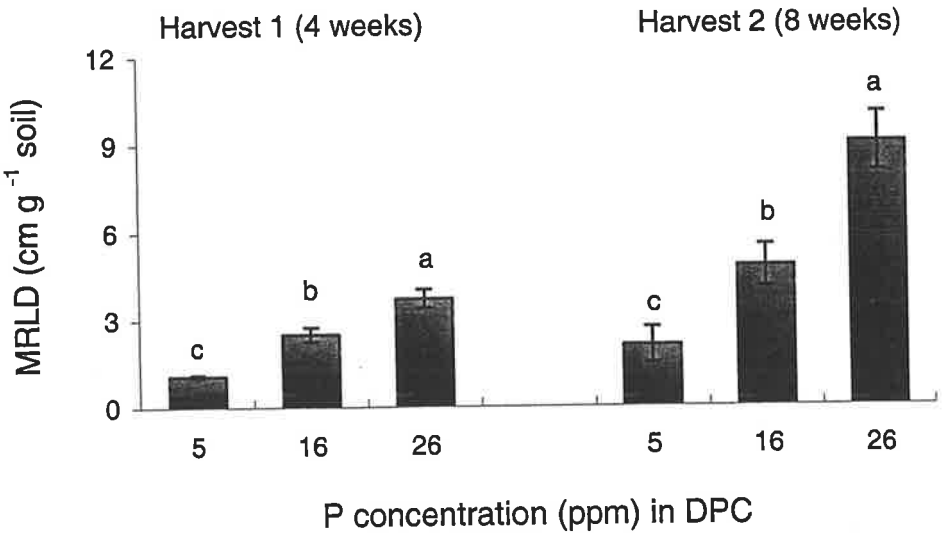

Figure 7.5 Effects of soil P concentration in DPC on mycorrhizal root length density (MRLD) of donor plants at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

7.3.3 External hyphal growth in HC

Fig. 7.6 shows different length density of external hyphae in HC in mycorrhizal inoculated pots with different available P concentration in DPC. Values have been corrected for the mean of values in equivalent uninoculated pots.

Growth of external hyphae of *Gi. margarita* in HC with a growth medium containing 11.9 ppm soluble Al was significantly affected by P concentration in DPC and varied with harvest time.

Hyphae growing from plants with P_5 in DPC had a relatively low length density, which did not change between H_1 and H_2. Increase in soil P availability from 5 to 16 ppm in DPC significantly increased hyphal length density in HC both at H1 and H2. However, further increase in soil P had no effect on the hyphae. There was no difference between P_{16} and P_{26} with respect to hyphal density either at H_1 or H_2.

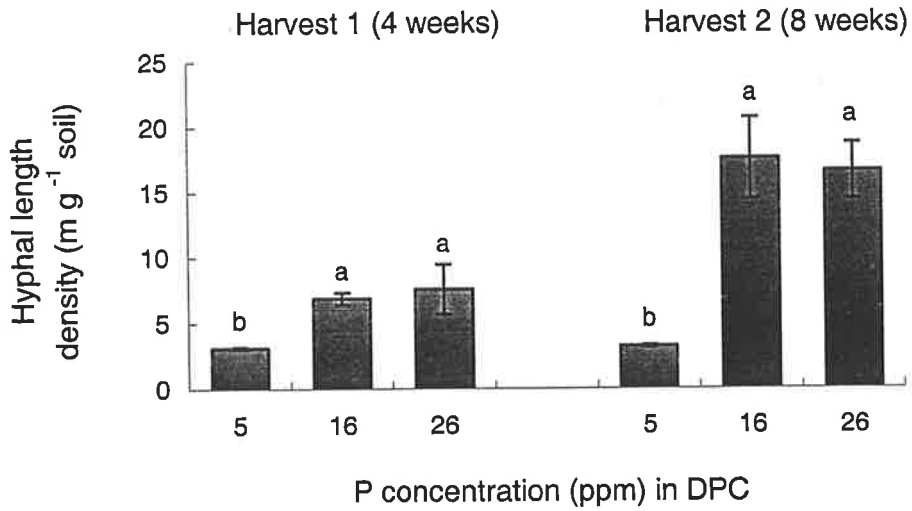

Figure 7.6 Effects of soil P concentration in DPC on external hyphal growth in HC at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

7.3.4 Root colonization of receiver plants

The roots of receiver plants in inoculated pots were colonized by hyphae traversing HC (no colonization was observed in uninoculated pots). However, the extent of the colonization was time-dependent and related to P treatments in DPC. At H_1, colonization was zero at P_5 or very low at P_{16} and P_{26}. Root colonization had increased sharply 4 weeks later for all plants, but to greater extent for P_{16} and P_{26} than for P_5. No difference was observed in root colonization between plants at P_{16} and P_{26} (Fig. 7.7).

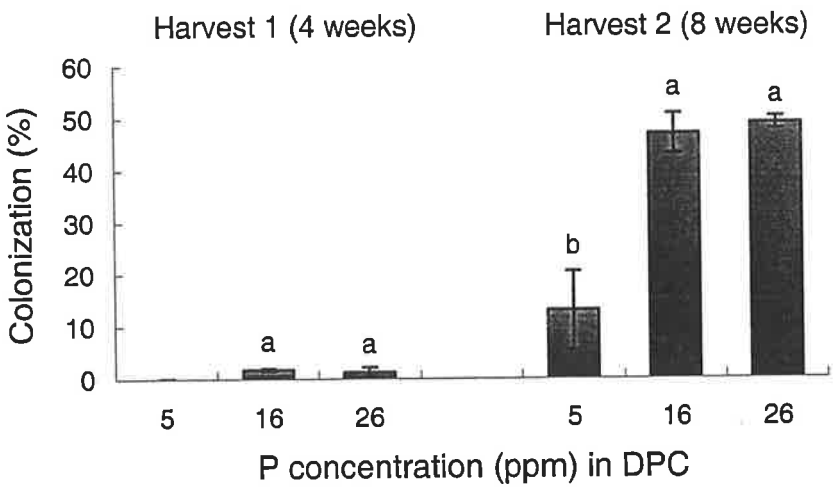

Figure 7.7 Effects of soil P concentration in DPC on mycorrhizal colonization of receiver plants at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

7.3.5 Receiver plant growth and P uptake

In contrast to plant growth in DPC, which contained different available P concentrations, growth differences in RPC with similar available P (26 ppm) were not observed at H_1. However, by H_2 the growth of mycorrhizal plants was higher than that of non-mycorrhizal plants particularly in pots with P_{16} and P_{26} in DPC (Fig. 7.8). There were also significant differences in growth of mycorrhizal plants with P treatments in DPC. Mycorrhizal plants in pots with P_{16} and P_{26} both had dry weights much higher than with P_5 but there was no difference between them. In pots without mycorrhizal inoculation, the plants in RPC grew similarly.

The same trend to plant dry weight was also observed for P uptake by mycorrhizal receiver and non-mycorrhizal plants in RPC (Fig. 7.9).

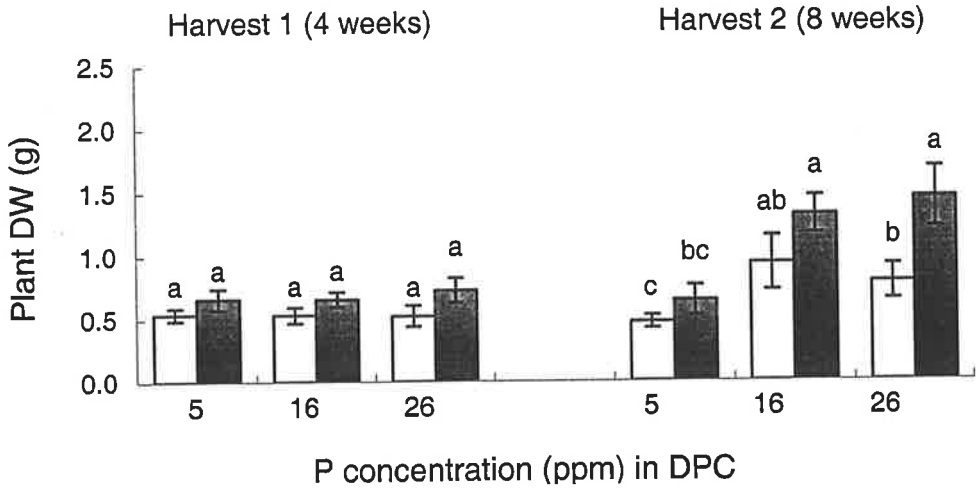

Figure 7.8 Effects of P concentration in DPC on dry weights of non-mycorrhizal (□) and mycorrhizal (■) plants in RPC at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

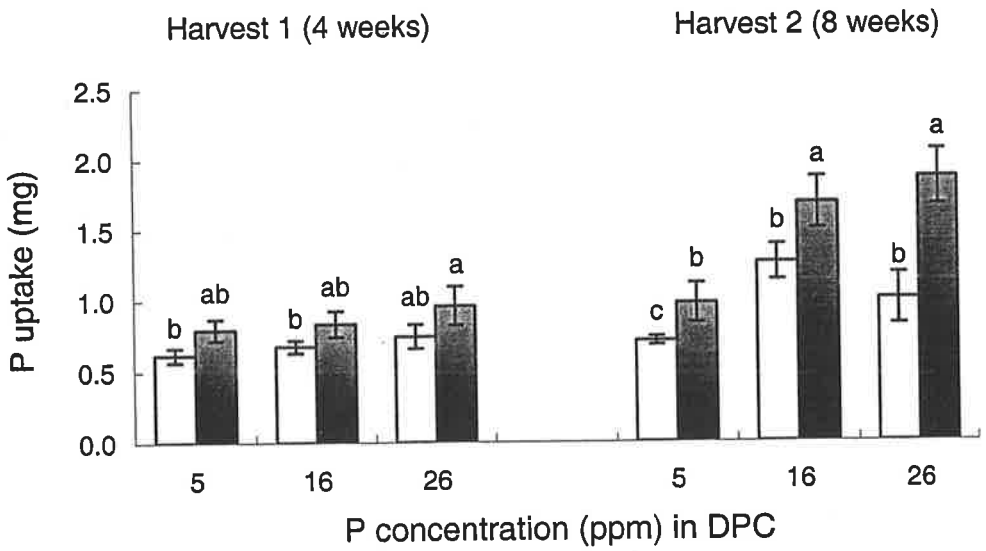

Figure 7.9 Effects of soil P concentration in DPC on P uptake of non-mycorrhizal (□) and mycorrhizal (■) plants in RPC at different harvest times

Bars in the same harvest group marked with the same letter are not significantly different at $P \leq 0.05$. Bars represent means and standard errors of means (n=3).

7.4 Discussion

This experiment investigated whether plant P nutritional status as a determinant of plant growth (Pairunan *et al.*, 1980) influences the growth of external hyphae of *Gi. margarita* in soil conditions containing 11.9 ppm soluble Al. This experiment tried firstly to establish differences in the growth rate of host plants (*here* donor plants) in compartment DPC by supplying the plants with different amounts of P, and at the same time maintain root colonization at approximately the same level. Figs. 7.1, 7.2 and 7.3 show that the expected differences in mycorrhizal plant growth, root length and P uptake induced by P treatments were apparent, with the trend of P uptake, root length and growth of plants following the concentration of available P in growth media. As a result, it is possible to suggest that variation in hyphal growth was caused by differences in the growth status of the host plants (Abbott *et al.*, 1984).

It was evident that the extension of hyphae growing out from host plants receiving different P supplies differed considerably between plants at low soil P (P_5) compared with those at medium and high soil P concentrations (P_{16} and P_{26}), particularly after 8 weeks. In P_5 plants grew poorly and hyphal length density was low. By contrast, when soil P was increased to 16 ppm, host plant growth increased significantly and hyphal density increased, although further increase in soil P concentration tended to reduce the hyphal growth. This finding indicates that increased available P in soil induced changes in the status of P nutrition and growth of host plant and

has considerable effects on the growth of external hyphae of *Gi. margarita*. It does not, however, always mean that the higher availability of P in soil may have greater effects on the hyphae as shown in Fig. 7.6; there was no difference in hyphal length density in P_{16} and P_{26} either in H_1 or H_2. This is consistent with a previous report for *G. fasciculatum* on subterranean clover (Abbott *et al.*, 1984), showing that at very low P availability both host plant and external hyphae grew poorly. Increasing available P increased both plant and hyphal growth but the optimal available P to produce maximum growth was different for plants and hyphae; when P was supplied to achieve maximum growth of the host plant, the growth of the hyphae decreased. It is possible that the poor development of external hyphae at very low soil P availability as shown here was due to lack of host plant growth resulting from decreased photosynthesis (Halsted and Lynch, 1996); decreased hyphal growth at high P (P_{26}) could be caused by decreased soluble carbohydrates within the roots (Jasper *et al.*, 1979).

A close correlation between hyphal length density in HC and mycorrhizal root length, but not percentage colonization (Table 7.1), indicates that the development of external hyphae was dependent on the development of internal colonization in roots. Increase in root length colonized in quantity might increase the number of colonization units (Abbott *et al.*, 1992) that in turn provides a larger root base for external hyphae to grow and spread in soil. In addition, the close correlation between hyphal growth and host plant growth and P tissue contents, but not P concentrations, indicates that the growth rate and P status of host plants might be important for the hyphae

because of their dependency on organic carbon from their host plants (Bago *et al.*, 2000).

This experiment also found that the external hyphae of *Gi. margarita* were, after delay, able to grow extensively through soil with the concentration of soluble Al as high as 11.9 ppm. Surprisingly, the length density of hyphae in this experiment was higher than values from Experiment 2 Section 6.3, in which lower Al concentrations were applied (ranging from 1.1 to 7.3 ppm). Although difficult to explain, this was possibly related to differences in plant growth influenced by day length. This experiment was carried out in December 2001 - February 2002 (summer), whereas Experiment 2 Section 6.3 was carried out 3 months earlier in September - November 2001 (spring) as described in Chapter 6.

Mycorrhiza formation that increases P acquisition and in turn enhances plant growth (*see* Smith and Read, 1997) was also found in this experiment. The growth and P uptake of donor plants was increased by mycorrhizal inoculation (Figs. 7.1 and 7.3). This was also observed for receiver plants after root colonization had become established at 8 weeks (H_2, Figs. 7.8 and 7.9). Importantly, this finding of increases in growth and P uptake by receiver plants following mycorrhizal establishment indicates that exposure to excess Al in the hyphal compartment did not affect the ability of external hyphae of the fungus to function both as inoculum for developing colonization (in receiver plant roots) and as an extended root system responsible for P uptake for the plants, as also observed in a previous experiment (Section 6.3).

Overall, it can be concluded that the growth and distribution of the external hyphae of *Gi. margarita* in the growth medium with excessive Al ions (about 12 ppm) was affected by the growth of host plants. Changes in the physiological conditions of the plants induced by available P in soil may affect the ability of the fungus to develop external hyphae in soil with Al stress.

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

As highlighted in Chapter 1 most terrestrial plant species, including many agricultural crops, form arbuscular mycorrhiza (AM) and get benefit from the symbiosis. In acid soils, formation of AM can be an important mechanism by which plants tolerate these soils (Sanchez and Salinas, 1981; Marschner, 1991). However, this may not always be the case, since AM fungi themselves may also be affected by adverse conditions in these soils. Soil acidity factors, excessive H^+ ion concentration and Al toxicity, might be major constraints for the functionality of AM symbiosis, but their potential effects have not been demonstrated separately (Siqueira *et al.*, 1984; Robson and Abbott, 1989; Habte, 1999).

The overall purpose of this study was to gain a better understanding of the behaviour of AM fungi in acidic soil conditions with a focus on the effects of Al on the development and function of AM symbiosis in cowpea plant growth.

In this last chapter the main findings already presented previously in Chapters 3 – 7 are discussed together in the light of research questions posed in Chapter 1 to draw conclusions and suggestions for future work.

8.2 Main findings

In general this study demonstrated that,

- Under acidic soil conditions either low pH *per se* or excessive soluble Al concentration could individually be serious constraints for the growth and function of AM fungi in plant growth (Chapters 3 and 4).
- Different fungal species differ in their responses to low pH. Decreased soil pH from 5.2 to 4.6 did not affect root colonization of *Gi. margarita* but reduced that of *G. etunicatum*, indicating a difference in pH preferences between the two fungi. In general *Gi. margarita* was more effective in increasing the growth and element uptake of cowpea at these pH levels compared to *G. etunicatum* (Chapter 3).
- Although *Gi. margarita* was acid tolerant, its symbiotic function in cowpea growth was reduced by the presence of high Al in the soil (Chapter 4). The ineffectiveness of the fungus was not related to percentage root colonization but appeared to be related to poorly developed external hyphae in soil (Section 6.2 Chapter 6). However, effects due to high Na added to control pH could not be discounted.
- Excessive Al ions *in vitro* reduced spore germination and germ tube elongation of *Gi. margarita* (Sections 5.3 and 5.4 Chapter 5). However, infectivity of the spores was not really affected by Al in soil; hyphae from germinated spores apparently continue to grow, with a possible stimulus coming from plant roots. Plants differing in P nutrition produced no different stimuli to the hyphae (Section 5.5 Chapter 5).

- Excessive Al ions in soil reduced the growth of external hyphae of *Gi. margarita* (Sections 6.2 and 6.3 Chapter 6), but did not affect the potential of the external hyphae to initiate new colonization and to function in plant growth as long as the plants themselves were not exposed to high Al (Chapters 6 and 7).
- The response of external hyphae of *Gi. margarita* to Al stress was greatly affected by the growth of host plants. Increasing available P and hence improving the plant growth increased the ability of external hyphae to grow in soil with high Al concentration (Chapter 7).

8.3 Discussion

The success of this project in separating effects of low pH *per se* from Al toxicity depended on the development of appropriate methods to set up a plant growth medium used in the experiments. The growth medium developed was a sand/soil (90:10) mixture set up to have different pH values with soluble Al at sub-toxic levels to cowpea plants, or to have different soluble Al concentrations at similar pH. Using the growth media the effects of low pH per se were successfully assessed separately from those of excessive soluble Al concentrations. This had not been achieved in previously published work because most investigations used soil for growing plants that could not be manipulated to separate the confounding effects of the two acidity factors. Therefore, the procedures to set up growth media developed in this study are potentially useful for ongoing studies that have a

requirement to establish soil conditions with specific element compositions for growing plants. A disadvantage is that preparation of the media is very time consuming, particularly with respect to establishing the dosage of elements that must be added to set up a certain soil condition; no standard recipes are available. Alternatively, semi-hydroponic sand cultures can be used, as in much previous work (Koslowsky and Boerner, 1989; Medeiros *et al.*, 1994; Mendoza and Borie, 1998; Rufyikiri *et al.*, 2000). However, this will produce different results (Yang *et al.*, 1996) due to differences in media conditions such as the contents of clay and organic matter that relate to pH-buffering capacity and Al solubility (Edmeades *et al.*, 1995). In addition, Drew *et al.* (2003), using single arm cross-pots, a type of compartmented pot systems, demonstrated that the development of external hyphae of *G. intraradices* and *G. mossae* was much lower in sand than in soil medium, indicating that sand culture might be less conducive for AM fungal growth and therefore unsatisfactorily for studies of AM function in acid soils.

The use of compartmented pots was also important in achieving the project aims. Previous studies on effects of AM fungi on plant growth in acid soils have used conventional (one compartment) pots, with the outcome that there are problems of distinguishing effects of soil pH on plants from effects on the external hyphae of the fungi (*see* Habte, 1999; van Aarle, 2002). The problem is exemplified in Chapter 4, describing the results of an experiment in which the plant roots and external fungal hyphae were both exposed to different Al concentrations. It was really difficult to determine whether inhibition of fungal growth and function resulted directly from toxic Al or was

related to depressed plant growth. Subsequently, this study used compartmented pots (Chapter 5 Section 5.5, Chapters 6 and 7). The use of different treatments or soil conditions for plant and/or for hyphae can be arranged in different compartments depending on experimental purposes. Hitherto, some aspects of development and function external fungal hyphae have been successfully studied using compartmented systems of different types (e.g. Schüepp *et al.*, 1987, 1992; Li *et al.*, 1991a, b; Jakobsen *et al.*, 1992a, b; Vierheilig *et al.*, 1995; Smith *et al.*, 2000; van Aarle, 2002). This study appears to be the first, in which compartmented systems have been used to study effects of Al on AM interactions, successfully distinguishing effects on plants from those on the external fungal hyphae. However, there may remain a problem of preventing solute movement from one compartment to another particularly for mobile elements such as Na and possibly Al. Future work should make particular attempts to maintain soil moisture contents in the different compartments of the pots, and also consider inclusion of buffer zones to prevent ion movements. Any such movements should be monitored by soil analyses. Despite the problem, many other eco-physiological aspects of AM fungi in acid soils can be studied effectively using compartmented pot systems rather than the conventional ones.

In general, this study clearly shows that both low pH in terms of excessive H^+ ion concentration (Chapter 3) and Al toxicity (Chapter 4) can independently affect the growth of AM fungi and their symbiotic function in plant growth. This finding is partly in line with some previous reports for the effects of soil acidity factors on the symbiosis (*see* Chapter 1 Section

1.2.2.2). However, the significance of this work is that it successfully distinguishes which soil acidity factor is mostly responsible for limiting mycorrhizal functioning in acidic soils.

Results of an experiment using growth media differing in pH (Chapter 3) showed that the two fungal species tested, *Gi. margarita* and *G. etunicatum*, had different responses to low pH. *Gi. margarita* was favoured by soil conditions of pH < 5.2, exhibited high ability to colonize plant roots at low pH and so improved the growth and element uptake of cowpea plants. In contrast, growth of *G. etunicatum* was limited by acidic soil conditions and the fungus might need a higher pH to be more effective. This finding is consistent with recent reports for *Gi. margarita* (Clark and Zeto, 1996a, b), but not for *G. etunicatum,* which was previously reported to function effectively in acidic conditions (Mendoza and Borie, 1998; Borie and Rubio, 1999). Differences in pH preferences among species and even isolates within species of AM fungi have been shown elsewhere (Bartolome-esteban and Schenck, 1984; Robson and Abbott, 1989; Clark, 1997) and together the results emphasize functional diversity of AM fungi in ecosystems (*see* Smith and Read, 1997). Because *G. etunicatum* lacks tolerance to low pH, this fungus was not used in further experiments dealing with Al toxicity that were carried out under soil conditions of pH 4.7.

Although *Gi. margarita* seems to be acid-tolerant, the results show that the presence of Al in soil can be a problem for its growth and function. Results in Chapter 4 obtained from an experiment using growth media containing soluble Al concentrations in the range of 1.1 to 11.9 ppm showed

that *Gi. margarita* was only effective in soil containing soluble Al at 1.1 ppm. The contributions of the fungus to growth of cowpea decreased with increasing Al concentrations. For instance, increasing Al from 1.1 to 7.3 ppm decreased mycorrhizal growth response from 85 to 21%, and P uptake response from 156 to 13%. This indicates that the fungus was unable to compensate for reduced growth of plant roots induced by Al toxicity. Excess Al also reduced the percentage of root length colonized by the fungus, but this reduction was not closely correlated with decreased mycorrhizal contributions to plant growth. These results, together with those presented in Chapter 3, suggest that *Gi. margarita* may have different responses to the two soil acidity factors, low pH and high Al. This fact, in general, may explain variation in AM functions in acid soils differing in pH and/or Al ion concentrations. Nurlaeny *et al.* (1996), for instance, found that *G. intraradices* had different effects on growth of *Zea mays* in two acid soils (similar in pH_{Ca} of 4.7) due to difference in Al concentrations. Clark (2002), using an acid soil rich in Al previously adjusted to the pH_{Ca} values of 4 and 5, found that *G. intraradices* was only effective in improving growth of *Panicum virgatum* at pH 5; in contrast, *G. clarum* was much more effective at pH 4 than at pH 5.

Reduction in root colonization and ineffective function of the fungus in this experiment were inconsistent with previous work showing that *Gi. margarita* was tolerant to Al toxicity (Bartolome-Esteban and Schenck, 1984) and made significant contributions to *Panicum virgatum* grown in an Al-rich acid soil (Clark *et al.*, 1999; Clark, 2002). Further investigations were therefore required to determine if decreases in root colonization (Chapter 4)

resulted from failure of primary colonization steps due to inhibition of spore germination and/or of germ tube growth or if the ineffectiveness of the symbiosis was related to inhibited external fungal hyphae by Al.

Spore germination and germ tube growth are critical phases in initiation of root colonization by AM fungi and are very sensitive to environmental stresses (*see* Siqueira *et al.*, 1985). Robson and Abbott (1989) suggested that low mycorrhizal colonization that often occurred in acid soils resulted in particular from inhibition of early growth of the fungi. Several studies using agar plates showed that either low pH or Al could inhibit both spore germination and germ tube elongation to variable extents, depending on fungal species (Green *et al.*, 1976; Siqueira *et al.*, 1982; Bartolome-Esteban and Schenck, 1984). In this study (Chapter 5) *Gi. margarita* was tolerant to low pH in terms of spore germination. At pH 4.5 about 40% of spores germinated and this increased with increasing media pH. The best pH (within the range tested) for germination of the fungal spores was around 5 (Fig. 5.2). Nonetheless, the presence of Al appeared to be detrimental for spore germination of *Gi. margarita* (Siqueira *et al.*, 1984). This was confirmed in this study, which showed that soluble Al at about 4 ppm in agar plates or in soil significantly decreased spore germination by about 50 and 25% respectively. Furthermore, the growth of germ tubes appeared more sensitive than spore germination both to low pH and Al toxicity. The growth was markedly inhibited at pH 4.6 compared with pH 5.3. The presence of Al at about 1 ppm in agar plates at pH 4.8 reduced the growth by about 30%, with further reduction to 70% at about 3 ppm. Therefore, decreases in root

colonization by *Gi. margarita* shown in Chapter 4 were probably the result of reduced germ tube growth in the soil, as well as reduction in the percentage of spores germinated. In consequence only small numbers of infective fungal propagules (spores in this case) would be available to penetrate the roots of plant and initiate colonization. Spores are significant propagules for *Gi. margarita*, and the ability of the spores to withstand excess Al is vital for establishment of colonization in acidic soil conditions.

The inhibitory effects of Al *in vitro* on germ tube growth were however not as pronounced in soil in symbiosis with plants. Results in Chapter 5 Section 5.5, obtained from an experiment using a trapping technique in which plants and spores were placed in different compartments, showed that soluble Al at concentrations up to about 12 ppm in soil did not inhibit germ tube infectivity. Hyphae growing from the germ tubes might continue to grow and colonize the roots of trap plants. This indicates that sufficient hyphae were in fact capable of tolerating Al toxicity.

The differences in toleration of excess Al by germ tubes in agar plates and in soil (Sections 5.3 and 5.5) could be related to higher solubility of Al in agar resulting in a stronger effect on the hyphae than in the soil. The presence of roots of trap plants might also contribute to the difference. Some workers demonstrated that root exudates have stimulatory effects on germ tube elongation (Gianinazzi-Pearson *et al.*, 1989) and hyphal branching (Paula and Siqueira, 1990; Nagahashi and Douds, 2000). In this respect, the quality of root exudates in terms of their stimulatory effects is closely related to plant P nutrition (Tawaraya *et al.*, 1998). Results of this study (Section 5.5

Chapter 5), in contrast, did not find any difference between cowpea plants differing in P status in stimulating hyphal growth. Another explanation for the difference is possibly related to the natural strategy of the fungus to survive under severe conditions. It is evident that germinating spores are able to extend hyphae for a few centimetres, but in the absence of host roots or failure to successfully establish symbiosis, hyphal growth is arrested within 1-2 weeks and the spores revert to dormancy (Bécard and Piché, 1989; Giovannetti *et al.*, 1993). Koske (1981) showed that AM fungal spores were able to re-germinate several times as long as their carbon supplies remained available, while Bartolome-Esteban and Schenck (1984) suggested that the extension of germ tube was limited by energy reserves in parent spores rather than severe conditions in soil.

Function of the external hyphae was investigated in an experiment using pots divided into compartments by 30 μm screen mesh. Plants with and without mycorrhizal inoculation were grown in a compartment (PC) containing soil without Al treatment (pH 5.3, with Al at sub-toxic concentration of 0.4 ppm). External fungal hyphae in association with the plants were allowed to grow into a root-free compartment (hyphal compartment, HC) that contained Al-treated soils. Results of the experiment in Chapter 6 Section 6.2 showed that the growth of external hyphae of *Gi. margarita* decreased with increasing soluble Al concentrations in soil. Although root colonization by the fungus was quite high (about 60%) regardless of Al treatments, increased Al concentrations in the hyphal compartment resulted in reduced plant responses to mycorrhiza, with the positive mycorrhizal growth response

disappearing when Al concentration was excessive in soil. These results clearly indicate that excess Al has detrimental effects on the external hyphae of the fungus and that mycorrhizal contributions under these conditions were dependent on hyphal function. Therefore, the ability of the fungus to develop an extensive hyphal network in soil appears to be a strong determinant for symbiotic effectiveness in this case, as already shown by Jakobsen *et al.* (1992a) and Li *et al.* (1991a, b).

The ability of hyphae of *Gi. margarita* to grow in soil containing high soluble Al was also apparent in a subsequent experiment using pots divided into 3 compartments; the two outer compartments were for donor (DPC) and receiver (RPC) plants and the other one in the middle was a root-free compartment for hyphae containing soil with different Al concentrations (Chapter 6 Section 6.3). The results demonstrated that external hyphae of the fungus had only a small response to excess Al in soil, similar to germ tube growth. Hyphae grew out from donor plants and traversed the hyphal compartment to colonize receiver plants in the RPC. If there was decreased hyphal length density with increasing Al concentrations in the HC it was more closely related to growth of the host plants than to direct effects of Al on the hyphae. The results also showed that exposure to high concentrations of Al in soil did not affect the potential for hyphae to colonize plant roots and to absorb and transfer mineral nutrients from soil to host roots. This is ecologically highly significant since the extent of colonization and symbiotic efficiency in acid soils in the field will greatly depend on the ability of the

hyphae to tolerate detrimental effects of soil acidity factors primarily high soluble Al in these soils (*see* Marschner, 1991; George, 2000).

Results of a subsequent experiment using the same pot systems (Chapter 7) showed limited development of hyphae from poorly-growing host plants when the hyphae were exposed to excessive Al of about 12 ppm in the hyphal compartment. The growth of the hyphae increased dramatically with increasing growth of the plants as a result of increased soil P availability from 5 to 16 ppm. However, further increased in soil P had no effects on hyphal growth. This is consistent with a previous report for *G. fasciculatum* in association with subterranean clover (Abbott *et al.*, 1984), showing that at very low P availability both host plant and external hyphae grew poorly. Increasing P supply increased growth of both plant and hyphae, but the requirement of P to achieve maximum growth differed between plant and hyphae. When P was supplied to achieve maximum growth of the host plant, the growth of the hyphae decreased. It is possible that the poor development of external hyphae at very low soil P availability was due to lack of host plant growth resulting from decreasing photosynthesis (Halsted and Lynch, 1996); decreased hyphal growth at high P could be caused by decreased soluble carbohydrates within roots (Jasper *et al.*, 1979). These factors clearly indicate that the growth and distribution of external hyphae were very dependent on the growth conditions of their host plants, particularly in relation to P nutrition.

8.4 Future work

In general, this study has demonstrated the ability of *Gi. margarita* to tolerate high Al in soil, but the mechanism has not been resolved due to time limitation. In particular, possible confounding effects of high Na, added in some treatments along with Al, should be resolved by titration with less potentially toxic alkalis (e.g. KOH). Organic acids such as citrate, malate and oxalate exuded by plant roots have been implicated in plant resistance to Al toxicity (*see* Jones, 1998; Ryan *et al.*, 2001; Hocking, 2001). Exudation of oxalic acid in particular by some ectomycorrhizal fungi has also been indicated (*see* Ahonen-Jonnarth *et al.*, 2000). Since no information is available for AM fungi it will be interesting to determine if the ability of *Gi. margarita* to tolerate Al was related to production of organic anions. In addition, Cuenca *et al.*, (2001) has indicated that Al was bound to cell walls in hyphae of AM fungi. Therefore, there is a need to confirm the capacity of Al binding by hyphae of *Gi. margarita* as another possible mechanism (Gadd, 1993) by which the fungus avoids Al toxicity. The use of monoxenic mycorrhizal root cultures in compartmented Petri dishes (*see* Bago *et al.*, 1996) and of fluorescent probes such as lumogallion and confocal laser scanning microscopy (Silva *et al.*, 2000) to detect Al^{3+} ions in fungal organs would be relevant in these studies.

It has been shown that the growth of hyphae from germinating spores is stimulated by the presence of host plant roots in axenic cultures (Gianinazzi-Pearson *et al.*, 1989; Nagahashi and Douds, 2000). The

stimulatory effect of cowpea roots, irrespective of P nutrition, on hyphal growth of *Gi. margarita* in soil was also indicated by results in Section 5.5 (Chapter 5). Plant species or genotypes may exude different amount of organic compounds (see Jones, 1998). Therefore, it is interesting to look at the effects of root exudates of Al sensitive and Al tolerant plant cultivars on stimulation of AM fungal growth.

In many tropical countries, multiple cropping is traditionally practised to sustain soil productivity (Beets, 1982). It includes intercropping with two or more crop species grown simultaneously on the same plot of land; crops may be intermingled or planted in separate rows or strips. In this system, crops of differing physiological responses to mycorrhizas and/or to Al might be included; and different behaviors of AM fungi can also be expected. For instance, the present study indicated that Al response of external hyphae of *Gi. margarita* differed when one or two plants were present in pots (Chapter 6). Therefore, it will be interesting to look at the growth and function of AM fungal hyphae when Al-tolerant and Al sensitive plants are combined. The possible function of external fungal hyphae in linking plants and so transferring nutrients between them could become a very interesting topic. Although complexity in interactions amongst symbiotic components can then be expected, the use of an appropriate compartmented pot system may make it simpler for these components to be assessed properly.

APPENDIX 1

Chemical characteristic of the soil and soil/sand mix used for the experiments

	Soil	[1]Soil/sand
pH_{H2O} (1:5)	4.9	5.3
[2]Organic C (%)	2.1	0.28
[3]Element (ppm)		
Ca	1615	324
Mg	1525	191
Na	635	345
K	4950	589
Al	10250	960
P	550	62
S	560	78
Fe	3950	340
Mn	89	8
B	585	61
Zn	57	16
Cu	97	<10
[4]Extractable-Al	68	5
[5]P-Bray 1	20	5
[6]Total N (%)	0.56	0.06

[1] Soil was mixed with sand in a 10:90 ratio;
[2] Organic C was determined using Walkley-Black method;
[3] Elements were extracted in a nitric/perchloric acid and determined by ICP;
[4] Al was extracted in 0.01 M $CaCl_2$ and determined by ICP;
[5] P was extracted in 1 M NH_4F (Bray-1 method) and determined colorimetrically;
[6] N was determined using Kjeldahl method.

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